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EMBRYONIC AND ADULT CARDIAC STEM CELLS - MOLECULAR, ELECTROPHYSIOLOGICAL AND IMMUNOLOGICAL CHARACTERISTICS FOR CARDIAC REPAIR

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"Education consists mainly of what we have unlearned"
Mark Twain

"The will to win, the desire to succeed, the urge to reach your full potential... these are the keys that will unlock the door to personal excellence"
Confucius

To My Great Family

CONTENTS

1 ABSTRACT

Background: The concept of myocardial regeneration by means of stimulating the endogenous regenerative potential *in situ* is an attractive approach. This offers distinct advantages to stem cell implantation where the problems with engraftment and immune rejection are avoided. Resident cardiac progenitors (CPCs) have emerged as promising optimal candidates for cardiac repair. The overall aim of this thesis was to identify and characterize the Isl1+ cells as promising CPCs; from the molecular, electrophysiological as well as immunological aspects in which human embryonic stem cells (HESCs) were used as a template for our planned transplantation studies.

Methods and Results: In *papers I-III*, based on both protein and transcriptional level analyses, we have identified the Isl1+ CPCs throughout the entire life span, both from the embryonic human heart and from the embryonic to the adult rat heart. Early in development the Isl1+ cells were mainly in the para-cardiac regions (pharyngeal foregut endoderm, splanchnic mesoderm, areas suggested to be the second heart field), while later in development they become predominantly localized in the following cardiac subdomains: outflow tract, inflow region of the right atrium and the upper part of right ventricle. Some of the Isl1+ cells were differentiating, while others were undifferentiated. However, only a minority of the Isl1+ cells was proliferating in contrary to the majority of the ventricular embryonic cardiomyocytes. After birth, immature Isl1+ cells were still present in the OFT where they resided until adulthood. Their distribution within the heart matched the defined embryonic distributions. Spontaneously beating *in vitro* cardiospheres were obtained from the embryonic human heart, exhibiting rate-response to electrical and pharmacological stimuli. To explore how cardiac regeneration and cell turnover adapted to disease, different forms of stress; physiological and pathological were studied for their effects on the CPC markers c-Kit and Isl1. Among the different stress modalities, ischemia-reperfusion (IR) injury was the strongest stimulus for activation of markers suggesting endogenous cardiomyocyte regeneration, correlating to the endogenous up-regulation of IGF-1 and HGF. There was a spatial mismatch on one hand of c-Kit and on the other hand Isl1 expression.
In *paper IV*, HESCs were used to test if the triple costimulation blockade regimen in a mouse model could induce a long-term immune tolerance to *in-vivo* transplanted HESCs to the testis and the heart. Costimulation blockade induced tolerance to undifferentiated HESCs in the immune-privileged environment of the testis and induced regulatory T-cells to HESCs when transplanted into the myocardium of immunocompetent mice. A booster dose of costimulation blockade induced HESC engraftment in one out of five immunocompetent mice.

Conclusions: The human embryonic heart is a potential source for the Isl1+ CPCs. These Isl1+ CPCs are present during the whole life span from the embryonic period until adulthood. They seem to have the capacity to differentiate into a cardiac specific-lineage. IR injury among other stresses was the strongest stimulus with both global and focal cardiomyocyte progenitor cell markers up-regulations in the adult heart. Short-term treatment with the costimulation blockade is sufficiently robust to induce long-term tolerance to transplanted HESCs in an immune-privileged environment and to induce regulatory T-cells when transplanted to the myocardium.

Key words: human resident cardiac progenitor, Isl1, c-Kit, Nkx2.5, IGF-1, HGF, ischemia-reperfusion injury, HESCs, costimulation blockade and tolerance.

2 LIST OF PUBLICATIONS

I. *Early first trimester human embryonic cardiac Islet-1 progenitor cells and cardiomyocytes: Immunohistochemical and electrophysiological Characterization*
Genead R, Danielsson C, Wärdell C, Kjaeldgaard A, Westgren M, Sundström E, Cereceda AF, Sylvén C, Grinnemo KH.
Stem Cell Research 2010; 4(1):69-76

II. *Islet-1 Cells Are Cardiac Progenitors Present During the Entire Lifespan: From the Embryonic Stage to Adulthood*
Genead R, Danielsson C, Andersson AB, Corbascio M, Cereceda AF, Sylvén C, Grinnemo KH.
Stem Cells and Development 2010; 19(10):1601-15

III. *Ischemia-reperfusion injury and pregnancy initiate time-dependent and robust signs of up-regulation of cardiac progenitor cells*
Genead R, Fischer H, Hussain A, Jaksch M, Andersson AB, Ljung K, Bulatovic I, Cereceda AF, Elsheikh E, Corbascio M, Edvard Smith C.I., Sylvén C, Grinnemo KH.
Submitted Manuscript

IV. *Costimulation blockade induces tolerance to HESC transplanted to the testis and induces regulatory T-cells to HESC transplanted into the heart*
Grinnemo KH*, **Genead R***, Kumagai-Braesch M, Andersson A, Danielsson C, Månsson-Broberg A, Dellgren G, Strömberg AM, Ekberg H, Hovatta O, Sylvén C, Corbascio M.
Stem Cells 2008; 26(7):1850-7

3 LIST OF ABBREVIATIONS

APC	Antigen presenting cells
CD	Cluster of differentiation
c-Kit	Tyrosine kinase receptor, stem cell factor receptor
CPCs	Resident cardiac progenitor cells
CS	Cardiosphere
CTLA4	Cytotoxic T-lymphocyte antigen 4
CTLA4 Ig	Cytotoxic T-lymphocyte antigen 4 immunoglobulin
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic cells
EB	Embryoid bodies
FHF	First heart field
FISH	Fluorescence in situ hybridization
FP	Field potential
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA binding factor 4
GD	Gestational day
HESC	Human Embryonic Stem Cell
HFib	Human fibroblasts
HGF	Hepatocyte growth factor
IBI	Interbeat interval
ICD	Implantable cardioverter defibrillator
IGF-1	Insulin- like growth factor-1
IHC / ICC	Immunohistochemistry / Immunocytochemistry
Isl1	LIM/homeodomain transcription factor Islet-1
LFA	Lymphocyte functional-associated antigen
MEA	Multi-electrode array
MHC	Major histocompatibility complex
mHC	Minor histocompatibility complex
MLR	Mixed leukocyte reaction
MSC	Mesenchymal stem cells
Nkx2.5	NK2 transcription factor related, locus 5
Oct	Octamer-binding transcription factor
PBS	Phosphate buffer saline
PSCs	Pluripotent stem cells
SCID	Severe Combined Immune Deficient mice
SD	Sprague-Dawley
SHF	Second heart field
SP	Side population
SSEA-1	Stage-specific embryonic antigen-1
SSEA-4	Stage-specific embryonic antigen-4
TnT	Troponin T
α-SMA	Alpha smooth muscle actin

4 INTRODUCTION

4.1 MAGNITUDE OF CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a disabling growing epidemic that causes premature death and decreased quality of life. CVD remains the leading cause of morbidity and mortality in the Western World, causing the death of an estimated 17.1 million people each year. By 2030, almost 23.6 million people are expected to die from heart disease and heart failure, according to the World Health Organization (Taylor et al., 2008).

Among the most common causes of CVD is ischemic heart disease (IHD) which if untreated properly may lead to the development of heart failure. With heart failure development series of gross and microscopic structural and cellular changes known as cardiac remodelling take place; among these changes the loss of the contractile cardiomyocytes is the dominant feature (Frangogiannis, 2008; Dobaczewski et al., 2010).

The human heart is comprised of about 2-6 billion cardiomyocytes (Olivetti et al., 1995), and other recent reports revealed that the heart of young adults from 17 to 30 years of age were determined to have an average of 6.0 and 2.2 billion cardiomyocytes in the left and right ventricular myocardium, respectively (Anversa and Olivetti, 2002).

Cardiomyocyte deficiency underlies most of the causes of heart failure. A 25- 30% infarct size could induce heart failure and the myocyte deficit in this infarction-induced heart failure corresponds to about one billion cardiomyocytes (Murry et al., 2006; Robey et al., 2008). A similar pattern was observed in other cardiac disorders such as hypertension as well as aging, in which a considerable loss of the cardiomyocyte mass occurred over years (Whelan et al., 2010; Olivetti et al., 1991).

Current approaches in the management of IHD and its implications; both medical and interventional, can only delay progression of the disease and the only standard therapy that addresses the fundamental problem of cardiomyocyte loss is heart transplantation. However, there are several problems and side-effects related to this procedure like donor availability, logistics, and transplant rejection.

The crucial issue is restoration and regeneration of the damaged myocardium, which remains a tremendous challenge, despite the major advances in this field. Cardiac regenerative stem cell-based therapy has emerged as a growing and promising approach for therapeutic myocardial regeneration and improvement of cardiac functions.

4.2 CARDIAC REGENERATIVE CELL-BASED THERAPY

Over the last decade, cardiac cell-based regenerative medicine has gone through exciting developments, starting with an initial phase of enthusiasm followed by a transient stage of suspicion whereas today regenerative medicine is going through major important steps to become clinically implemented.

One of these important steps is that we are continuously revising the findings from animal and human studies as regards which cell will be the optimal cell type for cardiac regeneration. By definition, the ideal cell type employed for cardiomyoplasty should be able to counteract the remodelling process as a part of heart failure development, which means that they should be able to generate new contractile cardiomyocytes, smooth muscle and endothelial cells to form new vasculature, and contractile myocardium. The

first cell type to be tested in clinical phase I trials was the skeletal myoblasts (Menasché et al., 2001).
In animal studies the myoblasts have proven advantageous since it was possible to use autologous cells. These could be expanded to millions of cells without changing their phenotype and after implantation they formed myotubes and improved left ventricular function (Hagège et al., 2003; Leobon et al., 2003). The first clinical phase I trials have demonstrated the feasibility of implanting autologous skeletal myoblasts concomitantly with coronary artery bypass grafting (CABG) and the patients improved symptomatically but developed problems with ventricular arrhythmias necessitating implantation of implantable cardioverter-defibrillators (ICD) (Hagege et al., 2006; Siminiak et al., 2004). The positive results on heart failure symptoms stimulated Menasché and co-workers to perform a multicenter, placebo controlled, randomized trial, the MAGIC trial which enrolled 120 patients. The results after 6 months were quite depressing since no significant improvement in cardiac function was observed. Instead they reported increased early postoperative arrhythmic events after myoblasts transplantation (Menasche et al., 2008). This trial ended the utilization of myoblast as a potential source for regenerating the heart.

Another set of cells that fuelled the field of cardiac regeneration were the bone marrow-derived somatic cells like the hematopoietic and mesenchymal stem cells. Quaini and co-workers in 2002 were first to demonstrate that in sex-mismatched cardiac transplants, a donor female heart transplanted into a male host contained Y-chromosomes in the cardiac cells, indicating their extra-cardiac origin (Quaini et al., 2002). Several positive animal studies have initiated enthusiasm to use autologous hematopoietic stem cells for regenerating the myocardium (Balsam et al., 2004; Jackson et al., 2001). One of these earlier studies was the study by Orlic and co-workers in 2001 (Orlic et al., 2001). In this study, bone marrow-derived Lin-/ c-Kit+ cells were shown to regenerate 68% of the infarcted area in a mouse model. These findings have been hard to be repeated by other groups (Balsam et al., 2004; Nygren et al., 2004; Terada et al., 2002; Murry et al., 2004) and according to the results by Nygren and other groups the effect exerted by these stem cells might be due to for example cell-fusion instead of transdifferentiation (Nygren et al., 2004; Alvarez-Dolado et al., 2003). Nonetheless, clinical trials using different subfractions of hematopoietic stem cells in patients with ischemic heart failure and chronic heart failure have been initiated.
Several clinical trials have used autologous cell populations. The first, randomized, controlled study was the BOOST trial (Wollert et al., 2004; Meyer et al., 2009), where bone-marrow mononuclear cells were delivered via intracoronary route 5 days after percutaneous coronary intervention (PCI). This study showed short but not long-term improvement of cardiac function. In contrast, the Repair-AMI trial (Schachinger et al., 2006; Assmus et al., 2010) reported a modest improvement of the left ventricular function at both 4 and 12 months follow-up. The results so far have not been so convincing and we still do not know if these cells actually develop into cardiomyocytes in humans, the mechanism through which these cells work, and if they have therapeutic paracrine effects.

The mesenchymal stem cells (MSCs) are different from the hematopoietic stem cells. These cells appear to be able to regenerate damaged myocardium both by exerting

paracrine effects, i.e. homing new cardiomyocytes and endothelial cells to the site of injury with induced angiogenesis (Mirotsou et al., 2007), but also by demonstrating a capacity to differentiate into cells similar to cardiomyocytes (Toma et al., 2002; Behfar et al., 2010). These cells contain contractile elements, but they do not contract spontaneously and they do not give rise to mature cardiomyocytes, not even after implantation to ischemic myocardium (Behfar et al., 2010). By directed cell expansion mesenchymal stem cells have been directed to a cardiomyocyte fate. These cardiopoietic mesenchymal stem cells have been tested in a clinical phase II trial and reported to induce physiological as well as clinical therapeutic efficacy in patients with chronic ischemic heart failure (Terzic et al, www.clincaltrials.gov) (Bartunek et al., 2011).

This means that none of the tested stem cells so far have been demonstrated to give rise to mature cardiomyocytes that can replace the damaged myocardium after ischemic injury. For this reason it is logical to continue the search for stem / progenitor cells found in the embryonic and adult heart, in order to use them as templates for the generation of stem cells for cardiomyoplasty. Another approach might be to promote existing cardiac stem cells and thus prevent progression to heart failure.

4.3 ENDOGENOUS REGENERATIVE POTENTIAL OF THE HEART

Several reports suggest that every adult mammalian tissue harbors a stem / progenitor cell population that could maintain or regenerate the host tissue in response to injury: bronchoalveolar stem cells (Kim et al., 2005), oval stem cells in liver (Gennero et al., 2010), and the quiescent satellite cell population that resides in a niche within the adult skeletal muscle, which becomes activated in response to severe injury (Meeson et al., 2007).

In the case of the heart, the regenerative potential seems to differ between different species. In metazoans such as newt and zebrafish, complex processes of cardiac regeneration have been revealed in response to injury (Bettencourt-Dias et al., 2003; Kikuchi et al., 2010; Jopling et al., 2010).

In mammals, for decades cardiomyocytes were seen as post-mitotic, lacking the capacity of self-repair after the neonatal period, eventually leading to heart failure if the myocardium was subjected to damage (Soonpaa and Field, 1997; Fernandez et al., 2001).

This old paradigm has been questioned over the last years. Even the adult heart seems to have an inborn regenerative capacity throughout life, probably from extra-cardiac sources where progenitors migrate and repopulate damaged myocardium, but this process occurs at very low rates (Laflamme et al., 2002). The adult heart is not a terminally differentiated organ but maintains limited regenerative capacity throughout life.

This notion is based on several studies in human and mouse using labeling strategies and genetic models. In humans, Bergmann and coworkers examined the cardiomyocyte turnover by measuring the radiocarbon (carbon 14) incorporation into the DNA (Bergmann et al., 2009). Nuclear bomb testing during the cold war increased the atmosphere concentrations of carbon 14, which subsequently was incorporated into the DNA of any living organism. By measuring the concentration of carbon 14 in different

organs, it was possible to get a quantification of cellular turnover and kinetics. This cellular dating technique and mathematical modeling support the notion that the adult human heart is capable of cellular turnover (representing cardiomyocyte renewal) at the rate of 1% per year at the age of 25 years, declining to about to 0.4% per year by the age of 75 years. This translates to that about 50% of the cardiomyocytes of an individual are being replaced during a life-time.
In another study, the rate of cardiac DNA synthesis was studied in post-mortem hearts from cancer patients treated with the thymidine analogue iododeoxyuridine, which incorporates into nascent DNA (Kajstura et al., 2010). Data revealed remarkably high rates of DNA labeling in cardiomyocytes, ranging from 2.5 to 46%.
Hsieh and co-workers used an inducible cardiomyocyte-specific transgenic fate-mapping (MerCreMer) strategy in the mouse, which allowed for the measurement of cardiomyocyte turnover in the adult mouse heart (Hsieh et al., 2007). These genetic studies were further supported by bromodeoxyuridine incorporation studies which supported the hypothesis that cardiomyocyte renewal occurred after myocardial injury

In summary, these studies support the notion that the postnatal mammalian heart has the capacity for cardiomyocyte renewal and turnover capabilities, which can be further increased after injury. However these elegant studies, do not explore the underlying mechanisms for this cardiomyocyte turnover, i.e. whether cardiac stem /progenitors were involved or not. In order to understand the endogenous regenerative capacity of the heart, it is mandatory to first go briefly through the concept of the embryonic heart development.

4.3.1 Embryonic cardiac stem / progenitor cells

4.3.1.1 Brief insight into the heart development

To date, most of the studies trying to explore the cellular and molecular mechanisms underlying the cardiac development have been based on murine models.
The heart is the first organ to develop in the embryo. The formation of the four-chambered heart from the pre-cardiac mesoderm into a well-developed pumping organ is a complex process. This process requires a diversity of cell types with specialized functions: cardiac muscle, endothelial, vascular, conduction cells, smooth muscle cells and interstitial fibroblasts (Vincent and Buckingham, 2010).
Recently, several studies have revealed increasing evidence that the diverse cell types in the heart are largely generated from multipotent cardiac progenitors arising from the early heart fields (Buckingham et al., 2005; Abu-Issa et al., 2004; Chien et al., 2008; Meilhac et al., 2004).
Two heart fields (HF), from which the embryonic heart develops, have been identified. These HF are defined as distinct embryonic regions in which cells that have myocardial potential are located (Abu-Issa et al., 2004). The earliest population of cardiac progenitors that comprises the first heart field (FHF) originates in the anterior splanchnic mesoderm giving rise to the cardiac crescent and subsequently the early heart tube and the left ventricle. Progenitors from the second heart field (SHF) that is derived from the pharyngeal mesoderm, anterior and medial to cells of the FHF, give rise to most of the cells of the right ventricle and the outflow tract (Abu-Issa et al., 2004; Buckingham et al., 2005).

As development progresses the heart tube forms and then undergoes looping to ultimately form the primitive chambers. The heart subsequently receives important contributions from two additional sources, the cardiac neural crest and the proepicardium. Cardiac neural crest cells contribute to normal development of the outflow tract and great vessels as well as important parts of the cardiac autonomic nervous system (Epstein and Buck, 2000; Hutson and Kirby, 2007); meanwhile the proepicardial cells migrate onto the surface of the heart, giving rise to the epicardium.
Little data is available as regards the transcriptional and molecular profile of the early heart development and most of our understanding is based on studies in animal models. To date, the cellular and molecular programming of the SHF cells has been better studied than the transcriptional regulation of the FHF cells.
The cardiac stem / progenitor cells that comprise the majority of the cells of SHF and seem to give rise to a major part of the developing heart (Cai et al., 2003), are the Isl1 progenitors. For this reason Isl1 cells are the main focus in this thesis.

4.3.1.2 Isl1 cardiac progenitors overview

Initial analysis of Isl1 null mice revealed an essential role of the transcription factor Isl1 in pancreatic and motor neuron development, and these mice died at embryonic stage E9.5 (Pfaff et al., 1996). Further studies revealed that Isl1 was reported as an insulin enhancer-binding protein expressed in cell lines of the pancreatic endocrine organ (Karlsson et al., 1990) and furthermore essential to control motor neuron differentiation during embryogenesis (Tsuchida et al., 1994; Thor et al., 1999).
Its importance for cardiac formation was discovered in early studies in chick embryos that revealed that the LIM–homeodomain transcription factor Isl1 was shown to be a marker of the heart field, that is expressed in undifferentiated cells in the cardiogenic mesoderm (Yuan and Schoenwolf, 2000).
Indirect lineage tracing experiments using the Cre-loxP strategy in mice have demonstrated that most of the cells of the early second heart field can be traced to multipotent heart progenitors that express the LIM-homeodomain transcription factor Isl1 (Sun et al., 2007; Moretti et al., 2006). Isl1 is transiently expressed in cardiac mesoderm, and although it is not strictly cardiac-specific, its expression has been used to identify cardiac progenitor cells. In addition *in vivo* cell lineage tracing in mouse embryos have confirmed that Isl1+ progenitors contribute to more than two-thirds of the cells in the embryonic heart including most of the cells of the conduction system, proximal coronary artery tree, atria, right ventricle, and outflow tract (Qyang et al., 2007; Cai et al., 2003). Cai and co-workers, have revealed that hearts of Isl1 null mice display severe cardiac defects, including complete absence of the outflow tract and right ventricle and partial loss of the atria (Cai et al., 2003).

A problem though is that most of the previous studies were based on indirect lineage tracing, retrospective clonal analysis, and transgenic models instead of studying the expression of the functional protein in order to identify the nature of these Isl1 cells. This means that cells actually expressing the Isl1 protein have not been characterized and usually there is a difference between gene transcripts and protein expression. Despite the fact that the Isl1+ cells are not fully characterized, they seem to be potent cells for regeneration of cardiac tissue. If these Isl1 cells are also present as progenitors in the adult heart or they disappear after birth is still unclear.

4.3.2 Adult cardiac stem / progenitor cells

Resident cardiac progenitor cells (CPCs) represent a responsive stem cell reservoir within the adult myocardium. These CPCs offer distinct advantages over the other circulating adult stem cells, like the bone marrow-derived stem cells. CPCs are precommitted to differentiate into cells of the cardiovascular entity, furthermore they are autologous and tissue-specific (Beltrami et al., 2003; Gonzales and Pedrazzini, 2009).
Adult progenitor cells that reside within the myocardium can be *autochthonous*, that is, leftovers of the embryonic life entrapped in the differentiated tissue, or *allochthonous*, that is, recruited from the bone marrow (or other organs) through the blood stream.

Many research groups have reported on different types of cardiac progenitor cells in different species with varying degree of cardiomyogenic potential and these cells exhibit stem cell-like properties, being multipotent and self-renewed.
These cells have been classified according to their properties and their expression of surface markers (Table 1), such as c-Kit (Beltrami et al., 2003; Dawn and Bolli, 2005), stem cell antigen-1 (Sca-1) (Oh et al., 2003; Matsuura et al., 2004), side population (Martin et al., 2004; Pfister et al., 2005), stage-specific embryonic antigen-1 (SSEA-1) (Ott et al., 2007), cardiospheres and/or cardiosphere-derived CPCs (Messina et al., 2004; Smith et al., 2007). Another source of adult CPCs with a great potential for regeneration is the epicardium (Cai et al., 2008; Zhou et al., 2008; Smart et al., 2007; Smart et al., 2011).

Cardiac progenitor cell type (CPC)	Marker profile	Frequency	Clonal activity	Differentiation potential
Lin-/c-kit CPCs	c-kit+, Lin-, CD45-, , Sca-1 ?, MEF- 2c low, CD34-, GATA- 4low, Nkx2.5+	1/10^4 cardiomyocytes ?	Yes	**CM, SMCs and ECs**
Side population (SP)	ABCG2+, c-kit low, Nkx-2.5-, CD45low, CD34low, GATA-4-	2% of total cardiac cells	Not determined	**CM, SMCs and ECs**
Lin-/Sca-1 CPCs	Sca-1+, c-kit-, CD34-, CD45-, GATA-4 low, Nkx-2.5-, MEF-2c+	1/3.3 x 10^4 cardiomyocytes	Not determined	**CM, SMCs and ECs**
CS /CDCs	c-kit+, CD31low, Sca-1+, cTnI low, CD34+, MHC+, Flk-1+, CD105+	10% of total cardiac cells	Yes	**CM, and ECs**
SSEA-1 CPCs	SSEA+, neonatal Nkx2.5 and GATA 4+ , adult OCT3/4+	?	?	**CM, SMCs and ECs**
EPDCs	Wt-1+, Tbx18+, Gata5+, some c-kit+ or Flk1+	?	Yes	**Fibroblast, SMCs, ? ECs and CMs**

Table 1. Phenotypic characterization of resident cardiac stem / progenitor cells
CM: cardiomyocyte; SMCs: smooth muscle cells; ECs: endothelial cells; EPDCs: epicardium-derived cells; CS/CDCs: cardiosphere / cardiosphere-derived cells.

In addition to the tissue committed monopotent or multipotent resident stem / progenitor cells (CPCs), there is growing evidence indicating that adult organs contain some other more primitive pluripotent stem cells (PSCs). One recent example is the

discovery of pluripotent stem cells known as very small embryonic-like (VSEL) stem cells, based on their small size, in the bone marrow, solid organs, umbilical cord blood and peripheral blood of mouse (Kucia et al., 2007; Ratajczak et al., 2008) and humans (Wojakowski et al., 2009). VSELs are non-hematopoietic expressing Sca-1+ /Lin− /CD45− as well as markers of pluripotency like Oct-4, Nanog, and SSEA-1 (mouse) and SSEA-4 (human). They can be differentiated into the three germ layers (Ratajczak et al., 2008). Wojakowski and his colleagues reported mobilization of VSELs expressing pluripotent markers, early cardiac and endothelial markers, and the chemokine receptor CXCR4 in patients with acute myocardial infarction (Wojakowski et al., 2009). The VSELs have been identified in the adult human myocardium expressing embryonic marker SSEA-4 (Wojakowski et al., 2011).

Although these cells hold a promising regenerative potential for cardiac repair, several challenges still remain to be clarified like their origin, the degree of overlap of the distinct sub-populations, their ability to retain their cardiogenic potential in disease or with aging. To which extent can their *in vitro* proliferative and differentiation potential be translated into a definitive *in vivo* beneficial effect on the injured tissue?

4.4 HUMAN EMBRYONIC STEM CELLS

The inborn regenerative capacity of the myocardium will not be enough to replace large areas of damaged myocardium. The endogenous cardiac progenitors may be suitable templates for the desired phenotype of the implanted cells. In order to generate sufficient quantities of these cells more immature cell may be needed, which can be expanded and differentiated *in vitro*. Human embryonic stem cells (HESCs) are prototypal stem cells, because they unambiguously fulfill all requirements of stem cells: self renewal, clonality and multipotency (Thomson et al., 1998).

Human embryonic stem cells (HESCs), which are isolated from the inner cell mass of the human blastocyst, can be propagated *in vitro* indefinitely, while still retaining their capacity to differentiate into almost all cell types. Their pluripotency has been proved by injecting undifferentiated HESCs into immunodeficient mice that resulted in teratocarcinoma including all three germ layers: endoderm, ectoderm, and mesoderm (Thomson et al., 1998; Reubinoff et al., 2000). Furthermore a variety of studies have described *in vitro* spontaneous and directed differentiation of HESCs to different lineages: neurons and glia (Carpenter et al., 2001; Reubinoff et al., 2001; Johnson et al., 2007), endothelial cells (Levenberg et al., 2002), hematopoietic precursors (Kaufman et al., 2001), trophoblast (Xu et al., 2002), hepatocyte-like cells (Rambhatla et al., 2003; Chen and Zeng, 2011), and cardiomyocytes (Xu et al., 2002; Kehat et al., 2001).

4.4.1 HESCs' capacity to generate cardiomyocytes

HESCs can probably be used in the future for cardiac repair, but then HESCs need to be differentiated into cardiomyocyte lineage. One approach to initiate differentiation of HESCs is to remove them from the fibroblast feeder layer and culture them in suspension. Then HESCs will aggregate into three-dimensional structures termed embryoid bodies (EBs). The aggregation process itself triggers cell differentiation. It is

thought that the EBs consist of derivatives of all three germ layers, and some of them start to contract spontaneously in culture (Itskovitz-Eldor et al., 2000; Doevendans et al., 2000).
Despite that these EBs harbor a panel of myocardial phenotypes based on transcriptional and protein analysis (Passier and Mummery, 2003; Gepstein, 2002), there will be a risk of teratoma formation upon *in vivo* transplantation (Kolossov et al., 2006). The conversion of undifferentiated ESCs into cardiomyocytes is still an inefficient process (Passier and Mummery, 2005; Laflamme et al., 2005; Lev et al., 2005; Kehat et al., 2001).
In order to produce more pure populations of cardiomyocytes with an adult molecular and electrophysiological phenotype (Sartiani et al., 2007), and thereby reducing the risk of teratoma formation, several strategies have been tried such as using ESCs in a monolayer culture, and co-culturing with either mature cardiomyocytes or visceral endodermal cells (END2) (Mummery et al., 2003) or using stimulating agents like 5-azacytidine, retinoic and ascorbic acid or growth factors like TGFß, FGF, IGF and PDGF families (Gepstein, 2002; Heng et al., 2004). Another approach is genetic manipulation involving cardiac specific promoters (Klug et al., 1996) or non-genetic approach using either mitochondrial fluorescent dyes (Hattori et al., 2010) or surface marker proteins such as cellular prion protein (Hidaka et al., 2010). This protein serves as an effective surface marker for isolating nascent cardiomyocytes as well as cardiomyogenic progenitors.

Induced pluripotent stem (iPS) cells expressing all the cardinal features of pluripotent stem cells have been generated by over-expressing four defined transcription factors (Oct3/4, Sox2, c-myc and Klf4) (Takahashi and Yamanaka, 2006) or with (Oct3/4, Sox2, NANOG and LIN28) (Yu et al., 2007) in several cell types, including fibroblasts.
Being autologous and patient specific, the use of iPS cells may overcome issues related to immunocompatability and bypass the ethical considerations associated with generation of ESCs. However, still controversial issues are holding back the clinical application of iPS. One of these issues is the heterogeneity in terms of plasticity and epigenetic labeling (Kim et al., 2010). Another issue is that the use of iPS poses the same risk of teratoma formation as ESCs, related to the impurity of the generated cell population. The human iPS cells can differentiate into various cell types, including cardiomyocytes (see review (Nelson et al., 2010)). However, the efficiency of cardiomyocyte differentiation is still not optimal. Applying the same strategies used for ESCs will improve the efficiency of cardiomyocyte differentiation before going into the clinic.
Even if these characteristics of the HESCs or iPS make them attractive sources of cells for cardiac repair there are other obstacles besides teratoma formation that must be considered before getting into the clinic. One vital issue is that HESCs are mainly derived from *in vitro* fertilization which means they are of allogeneic origin and will be rejected when transplanted into recipients. The immunogenicity of iPS cells has so far not been fully studied. In order to avoid this problem the immunogenicity of these pluripotent stem cells needs to be further characterized. In this thesis focus is only on HESCs. The immunological aspects of cell-based therapy will be highlighted through the next section, before further addressing the immunogenicity of HESCs.

4.4.2 Immunological aspects of cell-based transplantation

A major obstacle to bring allogeneic regenerative cell-based therapy into clinic will be avoiding the immune response towards the transplanted cells. This will subsequently require the long-term use of immunosuppressive regimens with its major drawbacks and side effects like cancer, renal insufficiency, osteoporosis, and hypertension.

The immune response can either be innate, adaptive or both. Rejection to transplants occurs due to differences in a multitude of antigens, which can be divided into three major groups: human ABO blood-group antigens, human leucocyte antigen/major histocompatibility complex (HLA/MHC), and the minor histocompatibility complex (mHC) (Bradley et al., 2002).
The ABO antigens consist of structurally highly polymorphic carbohydrate residues bound to cell-surface glycolipids and are expressed on almost every cell in the body (Clausen and Hakomori, 1989; Ito and Hirota, 1992). Transplantation of vascularised organs between ABO incompatible individuals will cause hyperacute rejection, mediated by preformed antibodies directed towards the ABO antigens, which activate the complement cascade (Paul and Baldwin, 1987; Cooper, 1990).
Another set of antigens are the HLA antigens, which are responsible for robust immunological rejection. They can be divided into HLA/MHC class I (HLA-A, -B, -C) and HLA/MHC class II (HLA-DR, -DQ and –DP) (Bradley et al., 2002). Most human cells express HLA class I molecules while the expression of HLA class II is restricted to antigen-presenting cells (APCs) such as dendritic cells (DCs), B-lymphocytes and macrophages (Viret and Janeway, 1999).
The mHC antigens are cell-derived peptides presented on the cell surface by MHC molecules. Snell and co-workers were the first to identify these antigens, on the MHC-identical donors (Snell, 1964). The graft rejection was slower than for MHC disparate mice and therefore these antigens were designated mHC. HESC express mHC and, although they are not as immunogenic as MHC and ABO antigens, they can nonetheless induce immune rejection (Simpson et al., 2001).
The immunological mechanisms that cause rejection of a cellular transplant can be divided into direct (Figure 1a) and indirect (Figure 1b) T-cell allorecognition pathways (Caballero et al., 2006). Under normal conditions, a T-cell receptor (TCR) recognizes a self-MHC molecule plus a foreign peptide and this leads to T-cell activation.
In direct recognition, the TCR recognizes the complex of peptide bound by an allogeneic MHC molecule and mistakes it for self-MHC with foreign peptide. In other words, an allogeneic MHC molecule with a bound peptide can mimic a particular foreign peptide, subsequently also inducing T-cell activation. The direct immune response will only be possible if a tissue that is transplanted contains donor APCs or if the transplanted cells can function as an APC. A prerequisite for functioning as an APC is the expression of MHC class II and of co-stimulatory molecules (Rogers and Lechler, 2001). After transplantation, the APCs from the donor tissue migrate to the regional lymph nodes in which they encounter and stimulate T-cells. The proliferation and differentiation of T-cells into activated T-cells require signals from both the TCR and co-stimulatory molecules (Figure 2a).
The B7 molecules are the classical co-stimulatory molecules expressed on the surface of DCs. These ligands bind to CD28 on the surface of T-cells and provide the second signal, which is a necessity for activating T-cells. Stimulation of naive T-cells by the

TCR without concomitant co-stimulation leads to a state of unresponsiveness called anergy (Frasca et al., 1998; Powell and Zheng, 2006). This is a state in which a T-cell is not only unresponsive on first encounter, but also remains so upon re-exposure. Some of these anergic T-cells develop into regulatory T-cells. These regulatory T-cells have the ability to inhibit the activation of new generations of naive T-cells with an affinity for the same antigens. In this manner, the immune system is capable of "learning and remembering". Previously activated effector and memory T-cells are less dependent on co-stimulation to get activated on second exposure than naive cells. Memory T-cells are responsible for the enhanced and accelerated immune response seen upon re-exposure to an antigen. The mechanism of memory T-cell generation and survival is a major field of investigation and difficulties in tolerance induction in these cells could well be the major obstacle to clinical tolerance induction (Kaech et al., 2002).
In the indirect T-cell allorecognition pathway, foreign peptides and allogeneic MHC molecules from the graft are processed and presented by a recipient APC. Processed MHC molecules will be recognized by T-cells like other conventional foreign protein antigens (Caballero et al., 2006). Indirect presentation results in allorecognition by CD4+ T-cells (helper T- cells). The alloantigens are acquired by the host APCs through the endosomal vesicular pathway (phagocytosis) and are presented by MHC class II molecules. Some antigens of phagocytosed graft material also enter the class I MHC pathway of antigen presentation and are recognized by CD8+ T-cells (cytotoxic T-cells) in a process called cross-presentation. Because MHC molecules are the most polymorphic proteins in the genome, each allogeneic MHC molecule may give rise to multiple epitopes, each recognized by different T-cells (Fairchild, 1998).

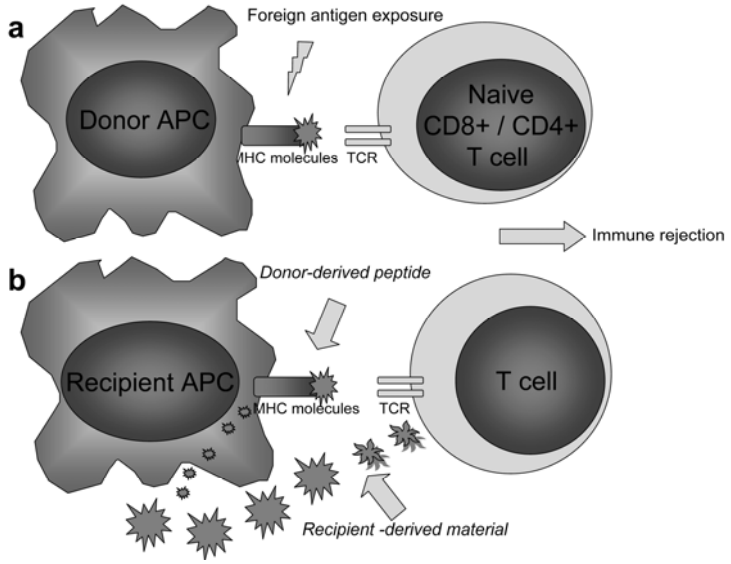


Figure 1. Antigen presentation.
a. Direct antigen presentation. Intact donor class-I and –II MHC molecules present on the surface of donor APC, which emigrate to the nearest lymph node, are recognized directly by T-cell receptor (TCR) on the surface of the recipient CD8+ and CD4+ T-cells, respectively. These T-cells interpret the donor MHC/peptide complex on the APCs as self-MHC with a foreign peptide, which induces T-cell activation and subsequently acute rejection.
b. Indirect antigen presentation. After implantation, donor antigen shed from the graft are internalized, partially processed by recipient APCs and presented as peptides to recipient T-cells. This will subsequently initiate T-cell activation.

4.4.3 The immunogenicity of HESCs

Whether HESCs are immune-privileged or not is another issue that must be addressed. It is not clear if HESCs and their differentiated progeny express ABO blood-group antigens but for clinical use this should be screened to avoid ABO incompatibility or using HESCs lines selected for the universal donor phenotype (blood group O).
What is clear is that HESCs express low levels of HLA class I, which is up-regulated by IFN-γ stimulation or after differentiation into embryoid bodies or after teratoma formation (Grinnemo et al., 2006; Drukker et al., 2002; Li et al., 2004). HLA class II molecules, however, are not expressed under these circumstances. The expression of co-stimulatory molecules (B7.1, B7.2 and CD40) has also been demonstrated to be low or absent on undifferentiated HESCs (Grinnemo et al., 2006; Li et al., 2004). This implies that the HESCs lack the two important prerequisites to function as professional APCs, namely high levels of HLA class II and co-stimulatory molecules.

To test the immune response evoked in a xenogeneic setting, Drukker and co-workers (2006) transplanted HESCs under the kidney capsule of various immunodeficient mice strains. Normal teratoma growth was only seen in the T-cell-deficient mice suggesting that xenograft rejection of HESCs was a T-cell-mediated immune process.
To confirm this outcome in an allogeneic setting, a trimeric mouse model was created in which the bone marrow was reconstituted with human peripheral blood mononuclear cells (human T, B and NK cells) (Drukker et al., 2006). Transplantation of human skin or a B cell lymphoma graft caused immune rejection in contrast to HESCs, which developed into teratoma.
In parallel Li and co-workers (2004) showed through *in vitro* studies using the mixed leukocyte reaction (MLR) that HESCs failed to induce proliferation of human peripheral blood mononuclear cells. This lack of allogeneic immune response was also seen when T-cell-enriched peripheral blood lymphocytes were used.
Based on these findings, Drukker and co-workers (2006) and Li and co-workers (2004) have previously suggested that HESCs are immune-privileged.
On the other hand, Grinnemo and co-workers have showed that injection of HESCs into the myocardium of immunocompetent C57BL/6 mice induced an immune rejection which peaked day 5 to 7 (Grinnemo et al., 2006). In a xenogeneic MLR, HESCs induced a similar immune response as human fibroblasts when co-cultured with mice CD4+ T-cells. Furthermore, lymphocytes isolated from mice previously transplanted with HESCs showed increased reactivity upon re-exposure to HESCs, indicating that immunological memory had been induced. In an allogeneic MLR, again HESCs and human fibroblasts induce a similar immune response when co-cultured with purified human CD4+ T-cells and DCs from the same donor. These HESCs induce a similar level of proliferation as human fibroblasts, without any sign of inhibiting proliferation. According to these data, HESCs are immunologically inert and do not inhibit immune responses.

4.4.4 Tolerance Induction

A hallmark of a normally functioning immune system is that a response is only directed towards foreign antigens. The avoidance of destruction of self-antigens is referred to as

self-tolerance. In order to induce tolerance to a transplanted cell, tissue or organ, we need to take both the innate and adaptive arm of immunity in account.

Strategies in transplantation across the immunological barrier

By reducing the alloantigen differences between host and recipient, transplantation of HESCs with a low risk of rejection might be possible. One possible solution would be the creation of special stem-cell banks, with cells having different HLA and ABO profiles that could be matched to the potential recipient. Another alternative might be to use somatic cell nuclear transfer (SCNT) (Wakayama et al., 2001; Wang et al., 2005) to create HESCs with antigen expression similar to that of the recipient. Another option might be to induce tolerance by co-stimulation blockade.

Immune tolerance is a state in which non-reactivity to an antigen is maintained in a host. In a healthy individual, this state is maintained in all the tissues of the body at a number of different checkpoints.

The most crucial step is the clonal elimination of T-cells with a strong affinity for self-antigens during the process of negative selection in the thymus. Some self-reactive T-cells might escape to peripheral immune tissues, where peripheral tolerance mechanisms control these potentially self-reactive T-cells. T-cell anergy refers to a functional state in which the T-cell becomes hyporesponsive following self-antigen encounter in the absence of optimal costimulation by APCs. T-cells chronically exposed to a self-antigen can also undergo apoptosis or develop into regulatory T-cells (CD4+CD25+Foxp3+), which actively suppress the activation and expansion of self-reactive T-cells.

In view of the adaptive and dynamic nature of the immune system, it should be possible to manipulate this system in order to induce tolerance. The blockade of costimulatory molecules that are necessary for optimal T-cell activation is an attractive strategy to induce tolerance to foreign antigens such as alloantigens after organ or cell transplantation.

Costimulation blockade has successfully been tested in rodents. The strategy that has attracted the greatest attention targets the co-stimulatory molecules CD40L expressed on activated T-cells and the B7 molecules expressed on DCs under inflammatory circumstances. These co-stimulatory molecules are expressed at the time of transplantation and in conjunction with the new antigen. This offers a temporal window in which tolerisation specific to donor antigen can be induced. Tolerisation is achieved by blocking CD40L with anti-CD40L and the B7 molecules with CTLA4Ig (a fusion protein of a high affinity receptor for B7 and the Fc component of human IgG (Figure 2b). The costimulation blockade is administered only during the first week after transplantation. The targeting of these pathways has lead to long-term acceptance of vascularised allogeneic and xenogeneic rat cardiac transplants in mice (Larsen et al., 1996; Elwood et al., 1998).

Depending on the type of tissue to be transplanted and the strain of mouse used as recipient, the outcome of tolerance induction with anti-CD40L/CTLA4Ig has been variable. This is attributed to the presence of CD8+ T-cells, which are resistant to double co-stimulation blockade (Trambley et al., 1999; Corbascio et al., 2002). By also targeting LFA-1, an important adhesion and co-stimulatory molecule for CD8+ T-cells, CD40L-independent immune activation and cytotoxicity can be inhibited. The addition

of anti-LFA-1 treatment to anti-CD40L/CTLA4Ig therapy has been shown to improve graft quality and function synergistically in discordant dopaminergic porcine xenografts transplanted into the brain of mice (Larsson et al., 2003). This therapy has also been shown to induce the permanent acceptance of porcine islet grafts transplanted into wild type diabetic mice (Kumagai-Braesch et al., 2007).

If this strategy is also applicable on undifferentiated HESCs is still unclear and should be explored.

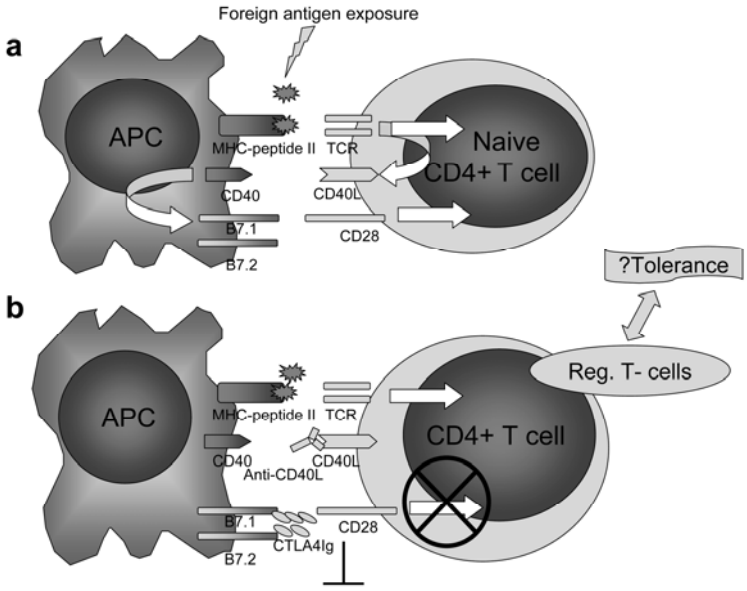


Figure 2. T-cell activation and peripheral tolerance induction
a. T-cell activation. The initial step in activation of naive T-cells is the binding of the T-cell receptor to a specific MHC complex on the membrane of an APC. CD40L binds to CD40 on the APC, enhancing the B7 expression on the APC and reinforcing the CD28 costimulation in a positive feedback loop.
b. Costimulation blockade and peripheral tolerance. This is achieved by treatment of transplant recipient with anti-CD40L which targets the CD40L expressed on activated T-cells and CTLA4Ig, which binds to the B7 (B7.1 and B7.2) molecule expressed on dendritic cells under inflammatory circumstances. This interaction delivers an inhibitory signal which means that the activated T-cell becomes anergic, upon a second exposure to the same antigen.

5 AIMS

The overall aims of this thesis were to explore the potential role of Isl1 progenitor cells for cardiac regeneration and to explore if immunologic tolerance induction can be used to get allogeneic stem cells into clinic.

The specific aims are listed according to the publications

I- To identify the nature of the Isl1 cardiac progenitor cells in the early first trimester human embryonic heart and to characterize the Isl1 cells in regard to their proliferation and differentiation capacity *in vivo* and *in vitro.* To immunohistochemically and electro-physiologically characterize the beating cardioshperes derived from the embryonic hearts.

II- To longitudinally characterize the distribution of cells actively expressing the cardiac progenitor transcription factor Isl1 during the embryonic life, the postnatal period, and adulthood in a rat model. To evaluate their proliferative and differentiating capacity *in vivo*.

III- To explore how stress can boost the endogenous cardiac regenerative potential, through the up-regulation of cardiac progenitor cell markers.

IV- To study the immunogenicity and tumorigencity of HESCs as a model for future differentiation into cardiac progenitor cells for cardiac repair. To study the ability of the costimulation blockade to induce long-term tolerance of the transplanted HESCs. To study the difference in engraftment between the testis and the myocardium.

6 MATERIALS AND METHODS

6.1 ANIMALS AND ETHICS

Time-mated and normal adult Sprague-Dawley female rats (Charles River, Germany) were used in *papers II and III*. Male C57BL/6 mice (B and K Universal AB, Sweden) and male SCID/Beige (C.B.-17/GbmsTac-SCID-bgDFN7; M and B, Taconic, Denmark) were used in *paper IV*.
All the procedures were approved by the Animal Care Committee of Karolinska University Hospital, Stockholm, Sweden and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health [NIH Publication No. 85-23, revised 1996].

6.2 PROCESSING OF HUMAN EMBRYONIC MATERIAL

In *paper I*, to collect human embryonic tissue, individual permission was obtained using a standard informed consent procedure and prior approval by the regional ethical committee. The investigation conforms to the principles outlined in the Declaration of Helsinki
In *paper IV*, human embryonic stem cells (HESC line HS401) were derived and cultured at the Fertility Unit, Department of Gynecology, Karolinska University Hospital following the approval by the regional ethical committee.

6.3 ANIMAL SURGERY AND HANDLING

6.3.1 Anesthesia and Post-Operative Care

In *papers III* and *IV*, both mice and rats were anaesthetized with intraperitoneal and subcutaneous injection of Midazolam (Dormicum, 5mg/kg) (Algol Pharma AB, Germany), Medetomidin hydrochloride (Domitor vet, 0.1 mg/kg) (Orion Corp., Espoo, Finland), Fentanyl (0.3 mg/kg) (B.Braun Medical AG, Seesatz, Switzerland), respectively. Mice were tracheomatized while rats were endotracheally intubated. The ventilation was maintained using a ventilator (model CWC600AP and 7025 Rodent ventilator, respectively, UGO BASILE S.R.L, Italy). The anesthesia was reversed by an intramuscular injection of Flumazenil (Lanexat, 0.1 mg/kg) (Hameln Pharma, Germany) and Tipamezol hydrochloride (Antisedan vet 5 mg/kg) (Orion Corp., Espoo, Finland). Postoperative analgesia was maintained by administrating Buprenorphin hydrochloride (Temgesic, 0.004 mg/kg/ twice per day for 3 days) (Schering-Plough Corp., Belgium). Doses were adjusted according to the weight of the animals.

6.3.2 Induction of myocardial ischemia in the rat model- Paper III

During general anesthesia a left thoracotomy was performed. In the myocardial infarction group the left anterior descending artery (LAD) was permanently ligated and induction of infarction was confirmed by color change and dyskinesia of the antero-lateral wall of the left ventricle. In the ischemia-reperfusion groups with or without growth factors, the LAD was temporarily ligated during five minutes followed by either

an intramyocardial injection of rrIGF-1(8µg) and rhHGF (2µg) dissolved in 10% rat serum in PBS or corresponding volume of only 10% rat serum, respectively.

6.3.3 Induction of immunologic tolerance of transplanted HESCs

Immuno-deficient SCID and immuno-competent C57BL/6 mice were chosen for analysis. The HESCs (200 000 cells) were injected either under the capsule of the right testis, an immunoprivileged organ (Nasr et al., 2005) or into the healthy myocardium (Figure 3). The mice were randomized to receive either costimulation blockade or isotype control reagents. The active costimulation blocking reagents used were anti-LFA-1 (clone M17/5.2), anti-CD40L (clone MR1), CTLA4 Ig, and their respective isotype control antibodies: rat IgG2b (clone 9A2), hamster IgG_1 and human IgG_1 (Bioexpress, West Lebanon, NH, USA).

Figure 3. An intubated C57BL/6 mouse with the heart exposed through a left open thoracotomy. HESCs were injected into the myocardium of the left ventricular wall.

6.4 CARDIOSPHERE CULTURE AND FREEZING- PAPER I

The rationale for using the human embryonic cardiosphere model was based on the notion that stem cells or cells with stem cell function will only retain their pluripotency within an appropriate environment, as suggested by the "niche" hypothesis (Nilsson and Simmons, 2004). This model has been studied in several cell types like neural cells developed into neurospheres (Galli et al., 2003), some tumor cell lines (LIM) (Bates et al., 2000), endothelial cells (Korff and Augustin, 1998) and fetal chicken cardiomyocytes (Armstrong et al., 2000). We aimed to test the possibility to reproduce the same model in our human embryonic heart material.

Human abortion material (gestational weeks 5 to 10) was transported directly from the operating room to the dissection room where the heart was the first organ to be identified to reduce the time of ischemia. Gestational age was determined by examination of the fetus for morphological landmarks (England, 1990). Time between abortion and culture preparation was 0.5–1 h.
For tissue culture, the cardiac material was first mechanically and then enzymatically digested repeatedly in collagenase solution (Collagenase type 2, CLS-2 Worthington 160 U/mL) until all pieces were completely dissociated. The obtained cells were washed by centrifugation and resuspended in knock-out Dulbecco's modified Eagle's medium (knock-out DMEM; Invitrogen, UK), nonessential amino acids (GTF,

Sweden), Primocin 100 µg/mL medium (Amaxa Inc., USA), 0.1 mmol/L β-mercaptoethanol (Invitrogen), glutamine 2 mM (Invitrogen), and insulin-transferrin-selenium supplement (Invitrogen). The cells were seeded directly onto plastic tissue plates (Techno Plastic Products AG, Switzerland) and cultured under serum-free conditions, in order to prohibit fibroblast growth, thus favoring cardiosphere growth. Freezing of the cultured cardiospheres was performed by detaching the cardiospheres and resuspending them in freezing medium [0.5 mL 20% knock-out serum replacement medium (Invitrogen) and 10% dimethyl sulfoxide (Sigma-Aldrich, USA) in knockout DMEM (Invitrogen). The cardiospheres were frozen gradually (−1 °C per minute) down to−70 °C and stored at−180 °C. When the frozen cells were recultured, they were quickly thawed to 37 °C, washed, and recultured on plastic plates.

6.5 IMMUNOSTAINING / DETECTION OF TRANSPLANTED CELLS

Immunostaining is a general term that applies to any use of an antibody-based method to detect a specific functional protein in a sample. This method was fundamentally applied to all our studies.

6.5.1 Immunohistochemistry / Immunocytochemistry

The information concerning all the used primary and secondary antibodies like dilutions; clone, company, etc. were described in details in the original papers.

In general, the used material was either paraffin-embedded or cryopreserved and sliced into 2.5-to 5-µm-thick sections. The paraffin-embedded sections were deparraffinized and frozen sections or cytospinned cells were fixed in 4% formaldehyde or ice-cold methanol, blocked with serum and incubated with the primary antibodies. The sections or cells were incubated with different fluorescence-labeled secondary antibodies (AlexaFluor 488 or 568 and TRITC). The stained cells were embedded in diamidino-2-phenylindole (DAPI) mounting medium and analyzed by fluorescence microscopy.
Enzymatic staining using horseradish peroxidase was applied in *paper II*, to achieve better overview of the cardiac and para-cardiac regions. This enzymatic reaction refers to a subclass of immunohistochemical procedures in which the antibodies were visualized via a peroxidase-catalyzed reaction. In brief, we used diaminobenzidine (DAB) as a color developer. Sections were counterstained with hematoxylin, mounted in a mounting medium and analyzed using bright field light microscopy. To achieve a proper antigen retrieval, the sections were boiled in 0.01 M citric buffer (pH 6.0), followed by 10 min incubation in 3% H_2O_2 to block endogenous peroxidase activity.

Various material and immunohistochemical analyses were used in the different papers: in *paper I*, the human embryonic hearts (5-10 weeks) and cultured cardiospheres; in *paper II*, whole rat embryos, which were sliced in a transverse plane from head to tail, together with early postnatal SD rat hearts (days 1–2) and hearts from adult SD rat; in *paper III*, adult SD rat hearts and in *paper IV*, the hearts and testes from SCID/ Beige and C57BL/6 mice.
In *papers I, II and III*, the hearts were mechanically minced into small pieces, treated with collagenase type II solution (CLS-2; Worthington Biochemical Corporation, Lakewood, NJ) to receive single cell suspensions for subsequent cytospin.

In *papers I and II*, the used material was analyzed for the expression of Isl1 as a marker for cardiomyocyte progenitor cells, Nkx2.5 as a marker to detect early differentiation into cardiomyocytes, Ki67 and histone H3 phosphorylation as markers for active cellular proliferation, and finally mature cardiomyocyte (troponin-T, TnT), and smooth muscle differentiation markers (α-smooth muscle actin, α-SMA).
In *paper II*, two different Isl1 antibodies were used, both goat anti-human Isl1 and mouse anti-rat supernatant Isl1
The goat anti-human Isl1 was used for immuno-fluorescence staining to be able to perform co-staining with differentiation and proliferation markers to avoid cross-reactivity with species of the same origin, while mouse anti-rat supernatant Isl1 was used for the immuno-peroxidase staining in paraffin-embedded sections because they give a better overview picture of the distribution of Isl1+ cells in the para-cardiac and cardiac regions. Both these primary antibodies were tested to stain the same rat Isl1+ cells thereby minimizing the risk of misinterpretation (Figure 4).

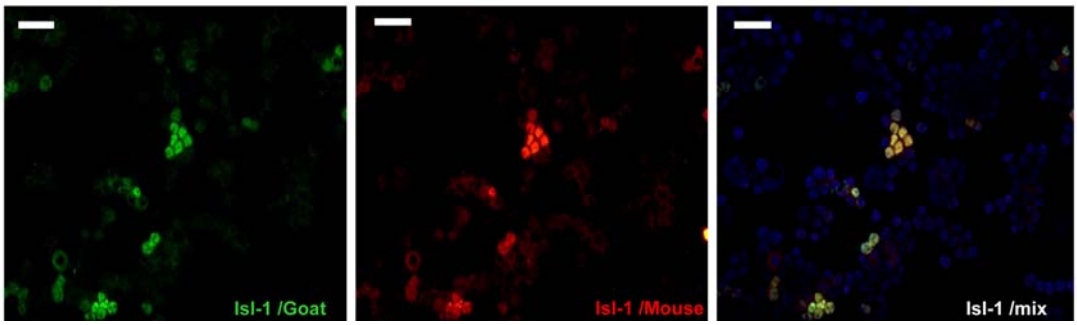


Figure 4. Fluorescent imaging showing the two different Isl1 antibodies used in *paper II* and their co-localization in the same cells which minimize the risk of misinterpretation.

In *paper I*, as positive controls, we used human fetal spinal cord (Isl1), human fetal heart (Nkx2.5), adult human atrial appendage (troponin T), and human fetal liver (Ki67) and as negative controls adult human spleen.
In *paper II*, the following positive controls were used: Isl1 (embryonic rat brain and spinal cord); TnT and α-SMA (fetal rat heart); Ki67 (fetal rat liver and intestine). For negative controls, the primary antibody was omitted and staining was performed with the secondary antibody on the same or the adjacent sections of interest.
In *paper III*, we used cytospin preparations of the adult rat hearts and stained for mast cells by using Toluidine blue staining.
Finally, in *paper IV*, freeze-sectioned hearts and testes were evaluated for the site of injection of HESCs via hematoxylin and eosin staining. In case of teratoma formation, these sections were further evaluated searching for the structures representing the three germ layers. The HESCs and their differentiated progeny were traced using fluorescence in situ hybridization technique (FISH) (described under 6.5.4 section). The sections with surviving HESC-derived cells in the hearts were further stained for the cardiomyocyte markers α-actinin and desmin, together with the marker for undifferentiated HESCs, TRA 1–60. As positive controls for the antibodies towards desmin and α-actinin we used sectioned human embryonic hearts.

6.5.2 Western blot / Immunoblotting

The Western blot (WB) analysis was used in *paper III* to be able to quantify the protein expressions of the target markers (Isl1, c-Kit and Nkx2.5) in comparison to the expression of the internal house-keeping control (GAPDH). WB is based on the principle that proteins are generally separated by size during the gel electrophoresis step and after blotting the studied proteins could be identified through comparison to known molecular weight markers.
In *paper III,* WB was used instead of immunohistochemistry (IHC) for two reasons: first, by using WB a more accurate quantitative estimation of the target markers (Isl1, c-Kit, Nkx2.5) among the different studied groups is obtained; second, interpretative difficulties related to background staining when using IHC on cytospin slides of adult rat hearts are avoided.

6.5.3 Transmission electron microscopy (TEM) - Paper I

TEM makes it possible to study tissues and cells at significantly higher resolution than with light microscope. This method was used in *paper I* where we wanted to characterize the beating cardiospheres at a sub-cellular level. Briefly, the spontaneously beating cardiospheres were collected with a micropipette and immediately fixed in 2% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (caco)/0.1 M sucrose/3 mM CaCl2 (pH 7.4) at room temperature for 30 min followed by 24 h at 4 °C. The specimens were rinsed in 0.15 M caco containing 3 mM CaCl2 (pH 7.4) and postfixed by incubation for 2 h at 4 °C in 2% osmium tetroxide in 0.07 M caco containing 1.5 mM CaCl2. The specimens were then dehydrated in an ascending series of ethanol followed by acetone and embedded in LX-112 epoxy resin (Ladd, Burlington, VT, USA). Semithin sections (0.5 μm) were placed on glass slides, stained with toluidine blue, and examined under a light microscope. Ultrathin sections (40–50 nm) were cut and contrasted with uranyl acetate followed by lead citrate and examined using a Tecnai 10 (FEI, Eindhoven, Netherlands) transmission electron microscope set at 80 kV.

6.5.4 Fluorescence in situ hybridization (FISH) - Paper IV

In *paper IV*, the HESCs and their differentiated progeny were traced using fluorescence in situ hybridization technique (FISH). This is a cytogenetic technique that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that only bind to those parts of the chromosome which show a high degree of sequence homology.
In our study, the aim was to detect DNA sequences specific for the human genome of the transplanted cells that would provide unequivocal genetic evidence for the accurate localization and identification of grafted cells in histological sections. These fluorescence inmarked DNA-probes that hybridized to the total human DNA of viable cells, labeled the whole nucleus.
In brief, the paraffin-embedded slides were deparraffinized. The samples were fixed onto cover slips, followed by denaturation of both fluorescent-labeled oligonucleotide probes and target DNA followed by overnight incubation to allow the probe to be hybridized with the sample. In the next step, excess probes were washed away, and fluorescence microscopy was used to view the location of the hybridized cells i.e. the implanted human cells.

6.6 ELECTROPHYSIOLOGICAL CHARACTERIZATION- PAPER I

6.6.1 Multi-electrode array (MEA)

The multi electrode-array method is well-described in numerous publications (Stett et al., 2003; Halbach et al., 2003). This MEA (Multi Channel Systems, Reutlingen, Germany) was chosen for an *in vitro* electrophysiological recording to allow collection of data from multiple sites in a non-invasive way in comparison to the patch-clamp method. Extracellular recordings were performed to characterize the basal electrical activity of the beating cardiospheres. Each MEA had 60 flat, round, substrate-integrated titanium nitride electrodes in an 8×8 grid and an integrated reference electrode (electrode diameter 30 µm and interelectrode distance 200 µm) (Figure 5). The MEA was connected to an amplifier with a heated stage (Multi Channel Systems, Reutlingen, Germany). All recordings were carried out at 37 °C with culture medium gassed with 5% CO_2.

Figure 5. Microelectrode array heating system and culture dish.

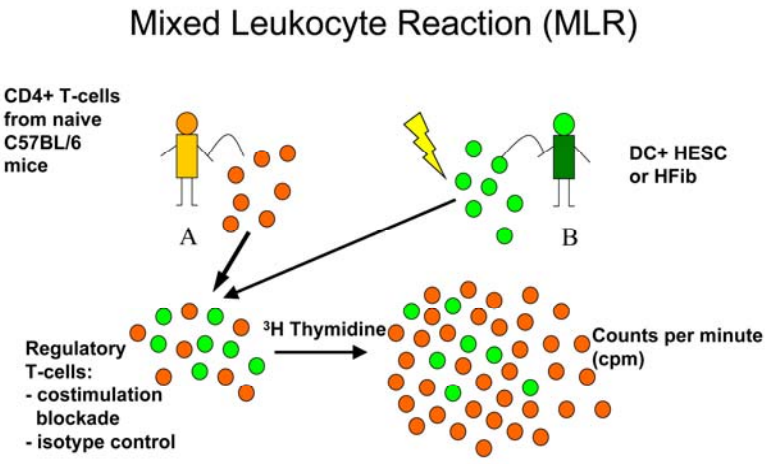
Spontaneously beating cardiospheres were selected and planted onto MEA plates pre-coated with human collagen IV (Sigma-Aldrich) or polyethyleneimine (PEI) + laminin. Two days after planting, the cells had attached to the plates, integrating mechanically and electrically, and forming large, simultaneously beating clusters covering several electrodes. Recordings were carried out within 5 days of planting.
Recordings were carried out at a sampling frequency of 1 kHz and lasted for 2 minutes. The resulting field potentials (FPs) were characterized regarding interbeat interval (IBI) and waveform properties such as FP_{MIN}, FP_{MAX}, and FP_{DUR}. Thirty successive FPs were averaged to reduce background noise and facilitate manual recognition of FP_{MAX}. To study the rate dependency of the action potential duration (APD), one cardiosphere cluster was paced with an external electrode in 100-ms steps at intervals between 1000 and 600 ms. The sensitivity to β-receptor stimulation was studied by adding 36 µM isoprenaline in the culture medium to the beating cardiosphere culture. Data were acquired and analyzed using MC Rack software (Multi Channel Systems).

6.7 IMMUNOLOGICAL CHARACTERIZATION- PAPER IV

6.7.1 Mixed leukocyte reaction (MLR)

The *in vitro* MLR method was performed to show if the *in vivo* immune reaction upon HESCs transplantation could be mimicked. The aim was to show if regulatory T-cells collected from mice treated with costimulation blockade could modulate the immune response induced by HESCs and whether this response is specific to HESCs or not.

Dendritic cells (DC) were prepared from the bone marrow of syngeneic C57BL/6 mice according to the protocol previously described (Grinnemo et al., 2006). Splenocytes from naive C57BL/6 mice, C57BL/6 mice from the costimulation blockade-treated and the isotype control groups were used for this experiment. The spleens were homogenized and the CD4+ T-cells were separated and CD4+CD25+ T-cells from C57BL/6 mice of the costimulation blockade and isotype control groups were separated. The DC were co-cultured with either irradiated HESCs or human fibroblasts (HFib). The MLR was performed exposing naive CD4+ T-cells to syngeneic DC alone or syngeneic DC co-cultured with HESCs or HFib. To study if the regulatory T-cells from mice treated with costimulation blockade or its isotype control reagents could specifically down-modulate the immune response induced by HESCs, the previously separated CD4+CD25+ T-cells were added to the wells. Each culture was labeled with 2µCi [3H] thymidine 20 h prior to harvest. The proliferation of the responders was measured at day 6 as counts per minute (cpm) and each experiment was performed in triplicate (Figure 6)



Schematic figure 6. Mixed leukocyte reaction (MLR): In brief, naive CD4 T-cells were exposed to syngeneic DC alone or DC co-cultured with HESCs or HFib. CD4+ CD25+ regulatory T-cells were added to the culture wells. Each well was labeled with radioactive reagent (3H) thymidine which allows the measurement of cell proliferation as counts per minute (cpm).

6.7.2 Histological evaluation of the immune response

The inflammatory response was evaluated by hematoxylin and eosin staining together with immunohistochemical analysis for CD3, CD4 and CD8 (markers of helper and cytotoxic T-cells, respectively, CD11b (activated macrophages) and FoxP3 (regulatory T-cells). Spleens from C57BL/6 were used as positive controls, whereas hearts from SCID/beige mice served as negative controls for the primary antibodies.

6.8 REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR) - PAPER III

The hearts were harvested through a left thoracotomy. The hearts were kept cold on dry ice and subsequently divided into the different sub-domains; outflow tract, right ventricle, left ventricle and when applicable the area of peri-ischemia and peri-infarct.

All the heart samples were snap-frozen in liquid nitrogen and kept in minus 70°C until RNA extraction. Total RNA was extracted from the heart samples according to the QuickGene RNA tissue assay (RT-S2, Science Imaging Scandinavia AB, Sweden) using the Fuji QuickGene-QG-810/QG-800Mini80 RNA isolation system. The RNA concentrations and purities (A260/280) were measured in a NanoDrop spectrophotometer® (ND-1000) (Nanodrop technologies, Wilmington, DE, USA). The RNA quality was further evaluated by 1 % agarose-gel electrophoresis.
Two micrograms of total RNA was reverse transcribed by Superscript reverse transcriptase (Life Technologies, Stockholm, Sweden) using random hexamer primers (Roche Diagnostics GmbH, Mannheim, Germany) in a total volume of 20 µl.
Real-time PCR was used to measure mRNA expression on a 7500 Fast real-time PCR system (Applied Biosystems Inc., Foster City, CA, USA). Primers and probes were supplied as a TaqMan® Reagents kit (Applied Biosystems). GAPDH was used as an endogenous control to correct for potential variation in cDNA loading.
All PCR reactions were performed in 96-well MicroAmp Optical plates (Applied Biosystems). cDNA was diluted 1:5. Amplification reagents (10 µl) contained 1 µl sample for c-Kit and Nkx2.5 and 5µl for Isl1 in TaqMan Universal PCR Mastermix.
All samples were run in duplicates. Each RT-PCR cycle consisted of: initial activation stage at 95°C (10 min), then the denaturation stage at 95°C (15 sec) and the annealing stage at 60°C (60 sec) cycled 40 times.
The comparative ΔCt method was used to calculate the relative gene expression to GAPDH for the genes analyzed (Winer et al., 1999).

6.9 STATISTICAL ANALYSIS

Data were presented as mean ± SD in all papers apart from *paper II* where values were presented as mean ± standard error (SE).
In *papers I and II*: Statistical heterogeneity was tested by Student's unpaired *t* –test.
In *papers III and IV*, Non-parametric (Mann-Whitney U) test was used.
In *paper III*: Statistical correlations were analyzed with the Pearson correlation test.
Statistical heterogeneities of $P < 0.05$ and $P < 0.001$ were considered to be statistically significant and highly significant, respectively.

7 RESULTS

7.1 PAPER I

Aims

To identify the nature of the Isl1 cardiac progenitor cells in the early first trimester human embryonic heart and to characterize the Isl1 cells in regard to their proliferation and differentiation capacity *in vivo* and *in vitro*. To immunohistochemically and electro-physiologically characterize the beating cardioshperes derived from the embryonic hearts.

Results

In vivo Isl1 progenitor cells distribution and characterization

In the early first trimester hearts (gestational weeks 5-9) Isl1+ cells were mainly clustered in the outflow tract and to a lesser extent in the atria and in the right ventricle. Some of the clusters were also expressing the cardiac contraction-related protein troponin T. Unexpectedly only few Isl1+ cells were Ki67+ while in the ventricles the majority of Isl1− troponin T+ cells were Ki67+ (Figure 7).

In vitro cardiosphere characterization

Cultures derived from the digested embryonic heart developed into spontaneously beating cardiospheres. At harvest, cells in these cardiospheres showed frequent expression of troponin T and Nkx2.5, while Isl1 was expressed only in scattered cells. Only a minority of the cultured cells expressed Ki67. The cardiospheres could be frozen, thawed, and recultured to beating cardiospheres. Electron microscopy revealed plenty of gap junctions between the cells in the beating cardiospheres together with contractile elements together with mitochondria and glycogen deposits, indicating metabolically active cells (Figure 8).

In the MEA system, autonomous interbeat interval (IBI) and field potential duration (FPdur) were determined from the beating cardiosphere clusters (1579 ms, SD = 633, range 923-2748 ms and 172 ms, SD = 58, range 110-265 ms respectively). Pacing in 100-ms steps at intervals between 1000 ms and 600 ms resulted in a rate-dependent decrease in FPdur of up to 35% at the highest stimulation frequency. β-adrenergic stimulation with isoprenaline shortened the IBI with 54% ($P<0.05$) and FPdur with 23% ($P<0.05$) (Figure 9).

Conclusions

The early first trimester human embryonic heart expressed clusters of Isl1+ cells; some were in the differentiation process, while others were in the undifferentiated progenitor form. Unexpectedly, only a minority of the Isl1+ cells, in contrary to the majority of ventricular cardiomyocytes, were proliferating. Spontaneously beating cardiospheres could be derived from the human embryonic heart and these cardiospheres were responsive to both electrical and pharmacological β-adrenergic stimulation.

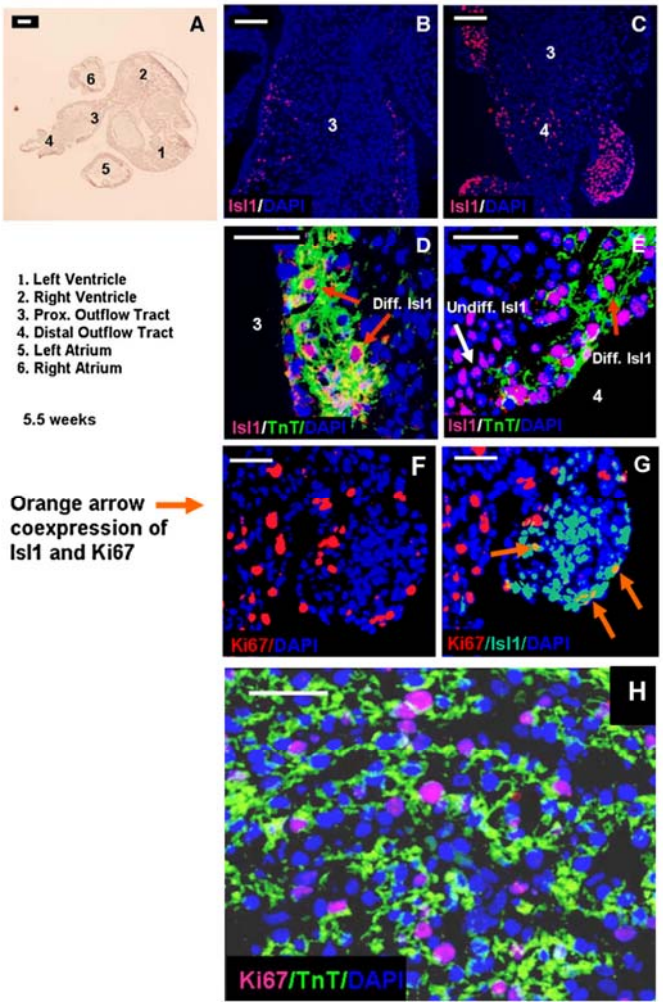


Figure 7. ***In vivo*** **characterization of a 5.5 weeks embryonic heart.**
Panel A: H & E staining of a 5.5 week embryonic heart delineating the different cardiac chambers.
Panel (B–E): The Isl1+cells were localized mainly in the proximal and the distal outflow tract. As shown in D and E, some of the Isl1+cells in the distal outflow tract were in the maturation and differentiation process expressing troponin T (red arrow), while some were in their undifferentiated form (white arrow).
Panels F and G: The Isl1 and Ki67 expression in human embryonic heart. Only scattered nuclei co-expressed Ki67 and Isl1.
Panel H: Human embryonic ventricular myocardium with co-expression of troponin T and Ki67.
Bars represent 200 µm in (A), 100 µm in (B and C), and 50 µm in (D- H).

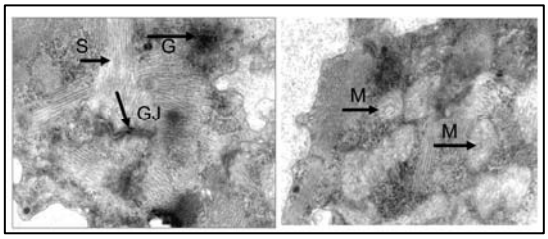


Figure 8. Electron microscopy of a beating cardiopshere revealed metabolically active cardiomyocytes with sarcomeric structures (S), mitochondria (M), and glycogen deposits (G), and that they formed gap junctions (GJ) with the surrounding cardiomyocyte. The contractile elements were not organized in the cytoplasm, a feature seen in cardiomyocytes that do not perform any work.

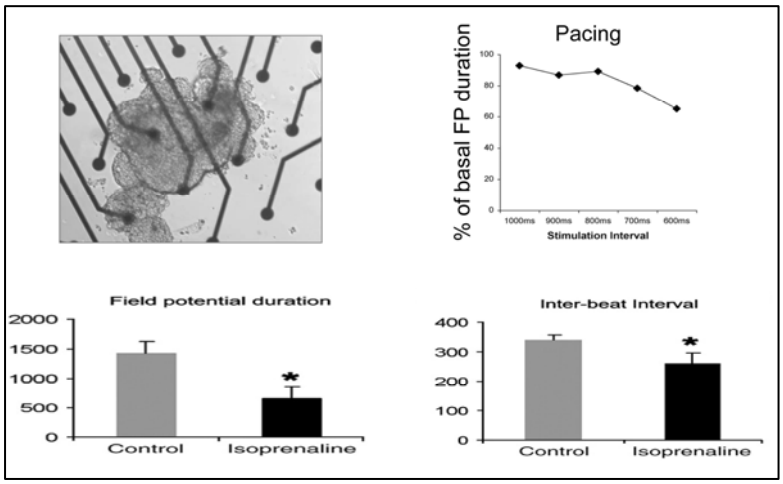


Figure 9. **Electrophysiological characteristics of beating cardiospheres**. Extracellular recordings of the beating cardiospheres were performed on a MEA culture dish (upper left). Cardiospheres were responsive to both electrical (upper right) and pharmacological β-adrenergic stimulation (bottom) FPdur and IBI data are presented as mean ± SD. * P<0.05.

7.2 PAPER II

Aims

To longitudinally characterize the distribution of cells actively expressing the cardiac progenitor transcription factor Isl1 in a rat model from the embryonic period to adulthood. To further evaluate the *in vivo* proliferative and differentiating capacity of Isl1+ cells.

Results

Distribution and characterization of Isl1+ cells in the embryonic heart from gestational day 11 to 15

The Isl1 expression in the upper part (outflow tract and atrial compartment) was 2.5 times higher than in the lower part (ventricular compartment) along the whole embryonic lifespan (Figure 11)**.** In early cardiac development gestational day 11 (GD 11), the Isl1+ progenitors were located in a greater abundance in the para-cardiac regions including pharyngeal foregut endoderm and splanchnic mesoderm, areas suggested to be the second heart field (SHF). To a lesser extent, Isl1+ cells were present within the bulbotruncal region and the truncus arteriosus. During the following days until GD 15, the Isl1+ cells were mainly observed in the proximal outflow tract and in the inflow area of the right atrium (Figure 10). No Isl1+ cells were detected in the left ventricle. Compared to GD 11, more Isl1+ cells seemed to co-express cardiomyocyte (TnT) and smooth muscle (α-SMA) markers and a minority of the Isl1+ cells was undifferentiated (Figure 12). Unexpectedly, only few undifferentiated Isl1+ cells were Ki67+ while a lot of TnT+ cardiomyocytes were proliferating in the ventricles (Figure 14). Our data suggests that during embryogenesis, Isl1+ cells migrate from extra-cardiac regions into the proximal part of the heart, proliferating and giving rise to cardioblasts. Unexpectedly, only a minority of the Isl1+ cells while a majority of ventricular cardiomyocytes were proliferating.

Distribution and characterization of Isl1+ cells in the postnatal and adult hearts

After birth, immature Isl1+ cells were still present in the outflow tract where they resided until adulthood. We show for the first time the existence of the Isl1+ cell pool in the adult hearts, localized within the myocardial layer of the proximal OFT as scattered cells, all expressing the cardiomyocyte marker TnT. No undifferentiated Isl1+ cells were observed in the adult heart (Figure 13).

Conclusions

In this systematic longitudinal study based on immunostaining during the embryonic morphogenesis of the heart, Isl1+ cells changed their distribution from extra-cardiac structures into the proximal part of the heart. Some of these cells were actively proliferating and part of the cells was in the process of differentiation and maturation co-expressing cardiomyocyte or smooth muscle cell markers. The Isl1+ cell pool persisted into adulthood.

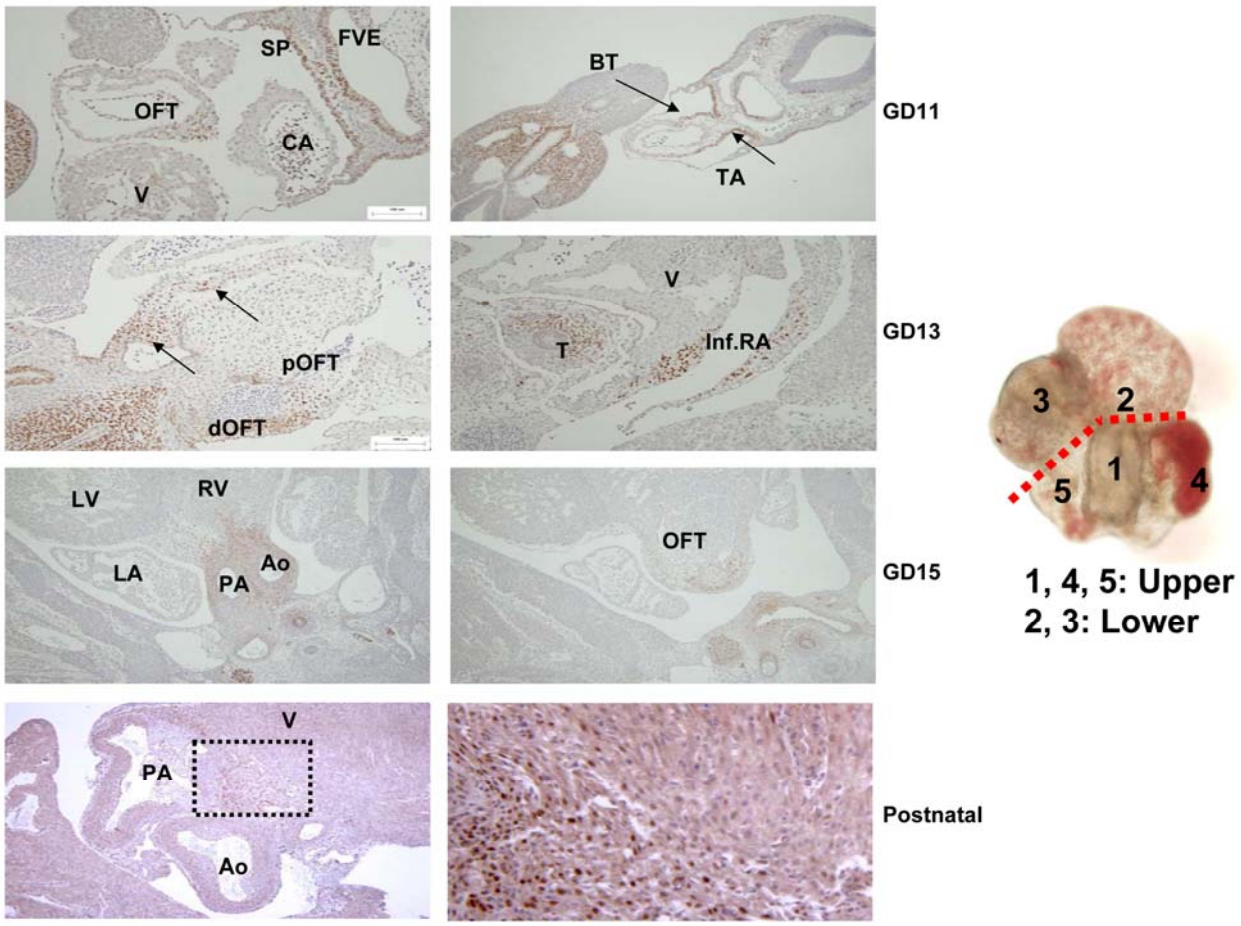


Figure 10. Isl1+ cell distribution from the early embryonic stage to postnatal life showing the dynamic Isl1 expression in both the cardiac and para-cardiac regions.
The present sections of the heart were sliced in a transverse plane from head to tail as shown in the schematic picture, where 1: outflow tract, 2: right ventricle, 3: left ventricle, 4: right atrium, 5: left atrium. Isl1+ cells (immunoperoxidase, HRP, brown cells). The section from the postnatal heart shows brown cells in the left part of the section. Abbreviations: OFT, outflow tract; LV, left ventricle; CA, common atrial chamber; SP, splanchnic mesoderm; FVE, foregut ventral endoderm; T, trachea; BT, bulbotruncal region; TA, truncus arteriosus.

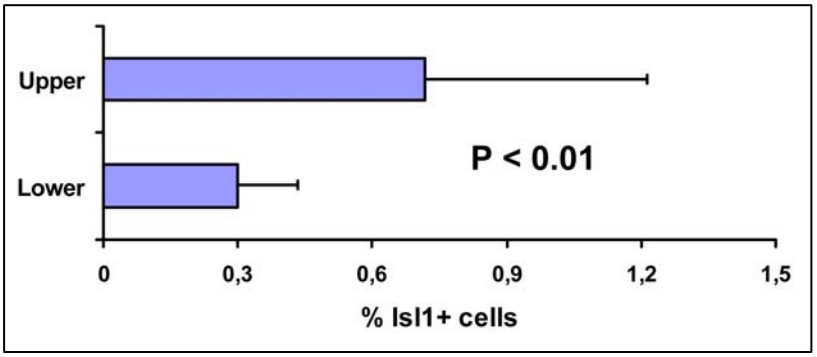


Figure 11. Distribution of Isl1+ cells in the upper and lower compartments of the developing heart.

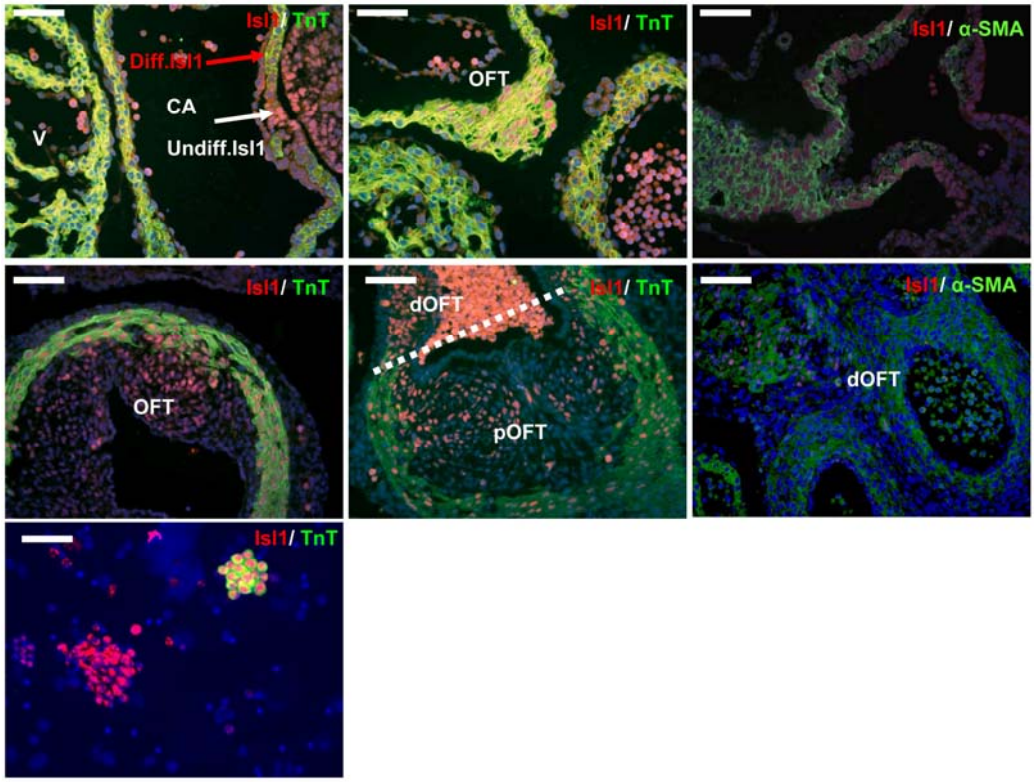


Figure 12. Isl1+ cell distribution, cardiomyocyte and smooth muscle differentiation in the cardiac and para-cardiac regions in the embryonic period (GD11-GD15).
Some Isl1+ cells are in the differentiated form expressing either TnT (Isl1+/ TnT+) (red arrow) or α-SMA, while other Isl1 cells are in the undifferentiated stage (white arrow). Data were shown both *in vivo* and in cytospin. OFT: outflow tract; CA: common atrial chamber; GD: gestational day.

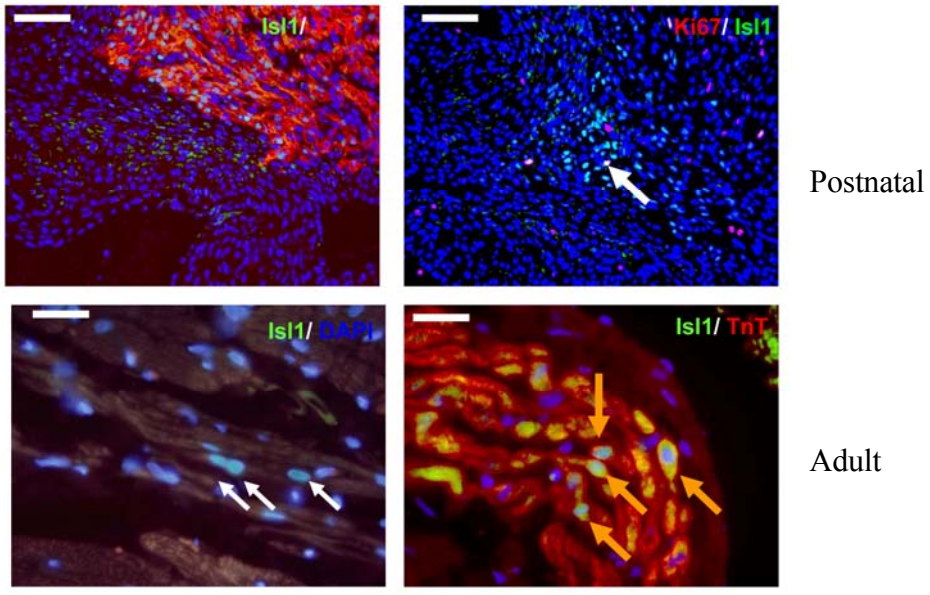


Figure 13. Distribution of undifferentiated Isl1+ cells together with cells proliferating and differentiating into cardiomyocytes at postnatal day 2 (upper panel).
In the adult heart (lower panel), the Isl1+ cells were localized within the myocardial layers of the outflow tract, all expressing the mature cardiomyocyte marker TnT (orange arrow).

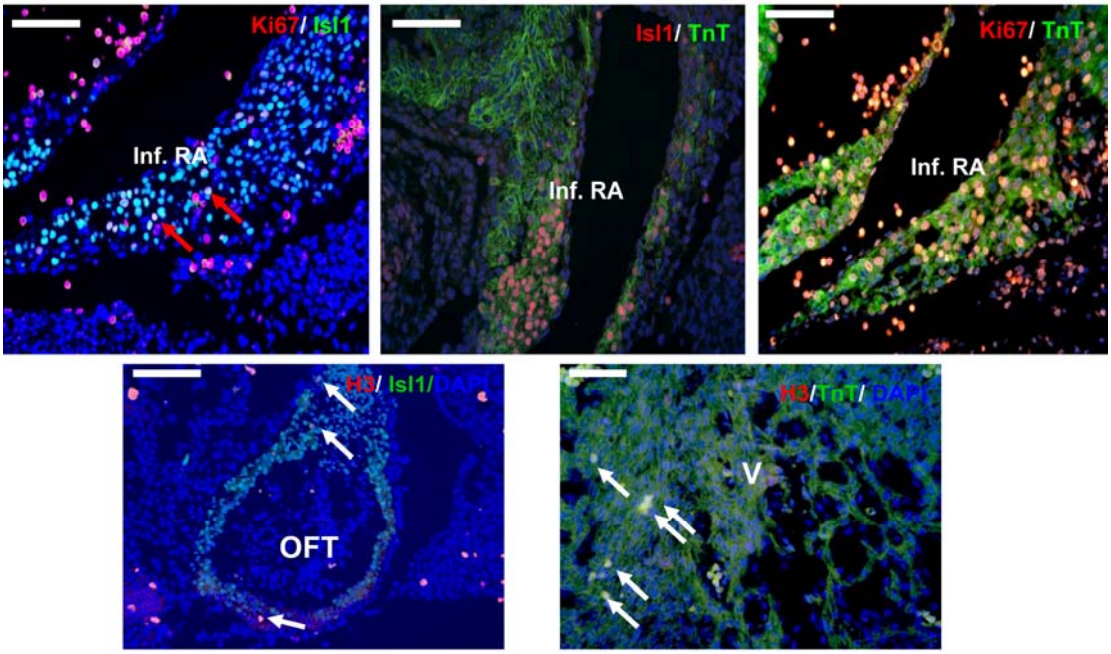


Figure 14. Distribution of actively proliferating Isl1+ cells and TnT+ cardiomyocytes in the embryonic heart.
Upper panel revealed that the majority of the embryonic cardiomyocytes (TnT+) express both Ki67 and Isl1 meanwhile only few Isl1 express Ki67 in the inflow part of right atrium (Inf. RA) (upper left, red arrows). Lower panel using histone H3 to confirm the proliferative capacity (M phase), it is evident that only few Isl1+ cells in the OFT undergo mitosis (left, white arrows) while the ventricular (V) cardiomyocytes have a much higher proliferative capacity (right, white arrows). OFT: outflow tract.

7.3 PAPER III

Aims

To explore how physiological and pathological stress could boost the endogenous cardiac regenerative potential, through the up-regulation of cardiac progenitor cell markers.

Results

The effect of ischemia-reperfusion injury, myocardial infarction and pregnancy on the expression of Isl1, c-Kit, and Nkx2.5

While pregnancy and myocardial infarction up-regulated Nkx2.5 and c-Kit (adjusted for mast cell activation) ($P<0.05$), ischemia-reperfusion (IR) injury induced the strongest matched up-regulation ($P<0.001$, $R= 0.524$) which occurred globally throughout the entire heart and not just around the site of injury (Figure 18).

Isl1 was not up-regulated by pregnancy or myocardial infarction but by ischemia-reperfusion injury ($P<0.05$), mainly in the outflow tract but also in the remote areas of the left ventricle (LV) and peri-ischemic regions (Figure 17).

Endogenous IGF-1 and HGF are involved in the up-regulation of cardiac progenitors

Endogenous up-regulation of IGF-1 was correlated to both increased c-Kit and Isl1 expressions ($P = 0.003$, $R = 0.235$ and $P = 0.005$, $R= 0.241$) (Figure 16). Endogenous HGF expression was correlated to increased c-Kit and Nkx2.5 expressions.

Furthermore the addition of IGF-1 and HGF in the IR group did stimulate their endogenous expression in the peri-ischemic region ($P<0.05$), which was also related to focal up-regulation of Isl1 in the OFT and the peri-ischemic region ($P<0.05$). c-Kit expression was not further influenced by the exogenous growth factor administration.

This indicates that there is a spatial mismatch between on one hand c-Kit and Nkx2.5 expression and on the other hand Isl1 expression.

Functional protein expression correlates with mRNA pattern

The mRNA expression patterns of c-Kit, Isl1 and Nkx2.5 were confirmed at the protein level at two weeks using Western blot analysis, where the highest protein expression of c-Kit, Isl1 and Nkx2.5 was observed in the IR injury groups (Figure 15).

Conclusions

Ischemia-reperfusion injury was the strongest stimulus for activation of endogenous cardiomyocyte regeneration, correlating to the endogenous up-regulation of IGF-1 and HGF. There was correlation between the up-regulation of both progenitor markers Isl1 and c-Kit and the up-regulation of the early cardiomyocyte marker Nkx2.5.

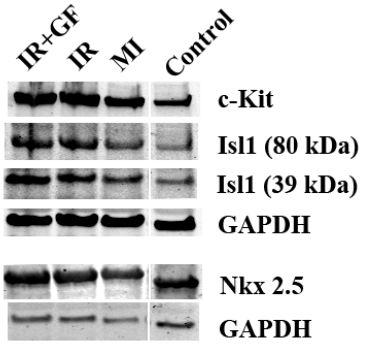


Figure 15. Western Blot analysis revealed the protein expression for c-Kit, Isl1 and Nkx2.5 at 2 weeks. Functional protein expression correlates with mRNA pattern where the highest protein expression was observed in the ischemia-reperfusion injury group.

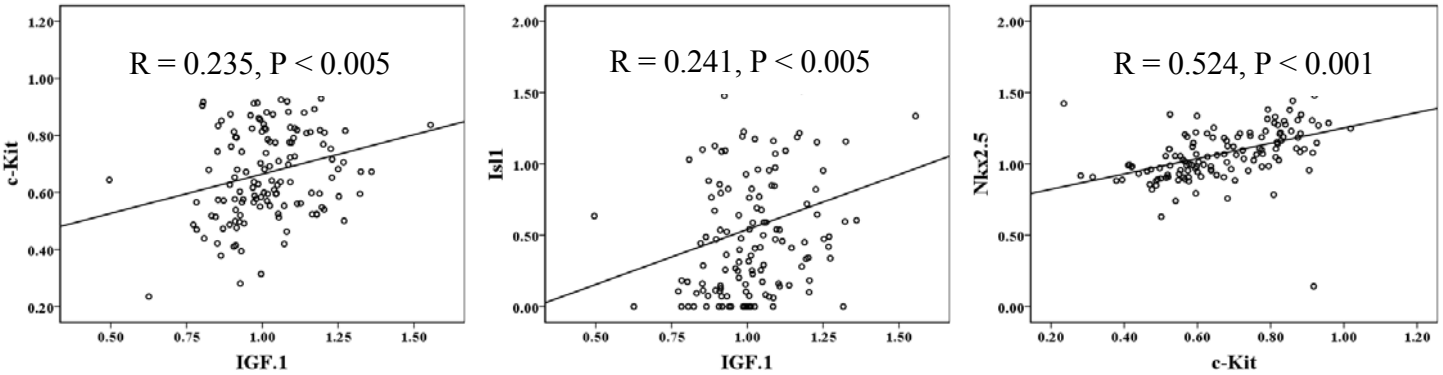


Figure 16. Scatter plots showing a strong positive correlation between the Nkx2.5 and c-Kit expression. Isl1 and c-Kit expressions were also positively correlated to the endogenous IGF-1 expression.

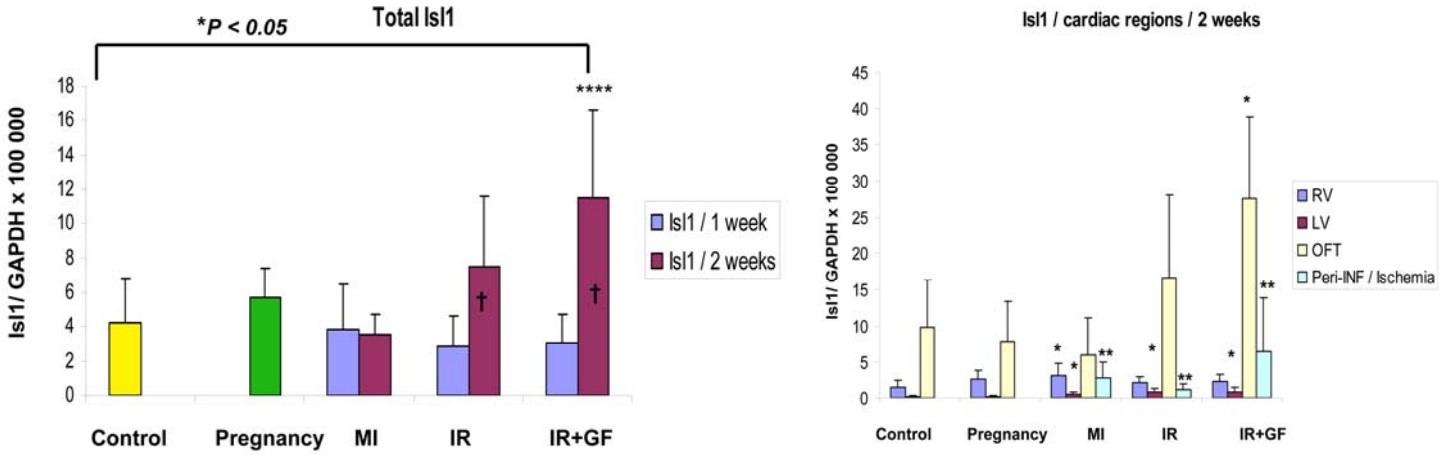


Figure 17. mRNA expression of Isl1 which revealed that ischemia-reperfusion injury induced a robust up-regulation compared to permanent myocardial infarction (left panel). This pattern of up-regulation was focally observed, primarily localized to the outflow tract and the right ventricle. The expression of Isl1 was also stimulated by IGF-1 and HGF, especially in the outflow tract area (right panel).

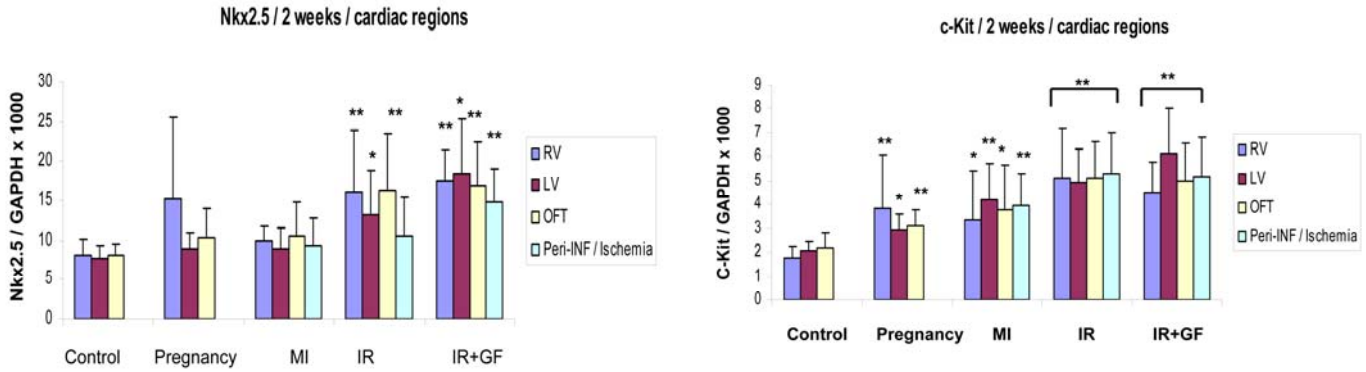


Figure 18. mRNA expression of Nkx2.5 and c-Kit at 2 weeks post-intervention in the different cardiac sub-domains.
Ischemia-reperfusion injury induced the strongest matched up-regulation. This up-regulation occurred globally throughout the entire heart and not just around the site of injury.

7.4 PAPER IV

Aims

To study the immunogenicity and tumorigencity of HESCs as a model for future differentiation into cardiac progenitor cells for cardiac repair. To study the ability of the costimulation blockade to induce long-term tolerance of the transplanted HESCs. To study the difference in engraftment of HESCs in the testis and the myocardium.

Results

In vivo histological analysis and immune response

All SCID mice with testicular HESCs transplants developed teratoma two months after implantation. The cells in the teratoma were of human origin (Figure 19). When SCID mice were transplanted myocardially, only two of five mice developed teratoma-like tumors (Figure 20). C57BL/6 mice with testicular transplants and treated with costimulation blockade all developed teratoma surrounded by CD4+CD25+Foxp3+ regulatory T-cells, while isotype control treated recipients rejected their grafts. C57BL/6 mice transplanted intra-cardially and treated with costimulation blockade rejected their grafts within one month, demonstrated by lymphocyte infiltrates one month after transplantation (Figure 21). Repeated costimulation blockade three weeks after implantation of HESCs into the myocardium of C57BL/6 mice caused survival of HESC-derived cells after two months (n=1/5) (Figure 22). The surviving cells were in-capsulated and there was no sign of inflammation.

Mixed leukocyte reaction (MLR)

MLR was designed to mimic the clinical setting of HESCs transplantation where the dendritic cells (DC) of the host present antigens from processed HESCs to host CD+ T-cells. In figure 23, the CD4+CD25+ T-cells separated from the costimulation blockade group significantly down-regulated naive CD4+ T-cell proliferation with a mean of 22% ($P < 0.05$), whereas CD4+CD25+ T-cells from the isotype control group did not inhibit proliferation of naive T-cells. Furthermore, the down-regulatory effect of the immune response mediated by CD4+CD25+ T-cells was specific to undifferentiated HESCs, since they did not inhibit the proliferation of naive T-cells stimulated by HFib.

Conclusions

Costimulation blockade is sufficiently robust to induce tolerance to undifferentiated HESCs in the immune-privileged environment of the testis and to induce regulatory T-cells to HESCs when transplanted into the myocardium of immunocompetent mice. This peripheral tolerance seems to be mediated by HESCs specific regulatory T-cells.

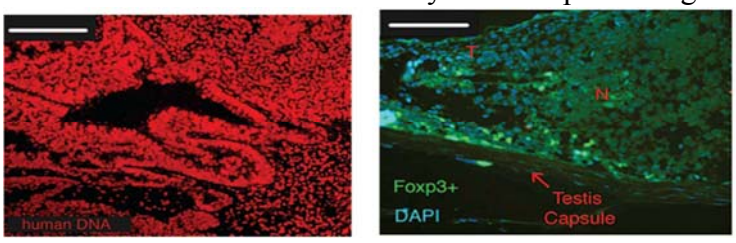
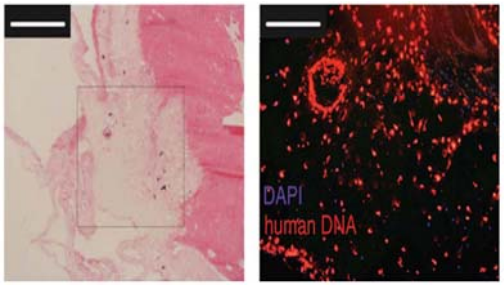


Figure 19. HESC-derived teratoma in the testis of a costimulation blockade treated C57BL/6 mouse 2 months after injection. The teratoma was of human origin; all cells were FISH positive (red cells) (left panel). CD4+CD25+Foxp3+ T-cells (green cells) were detected at the interface of normal tissue and teratoma in the testis (right panel). Bars represent 200 μm and 100 μm in left and right panels, respectively.

Figure 20. Left panel: Hematoxylin and eosin staining of an expansive growing teratoma-like tumor in the heart of a SCID mouse
Right panel: FISH staining reveals that the tumor originates from the HESCs (red cells).



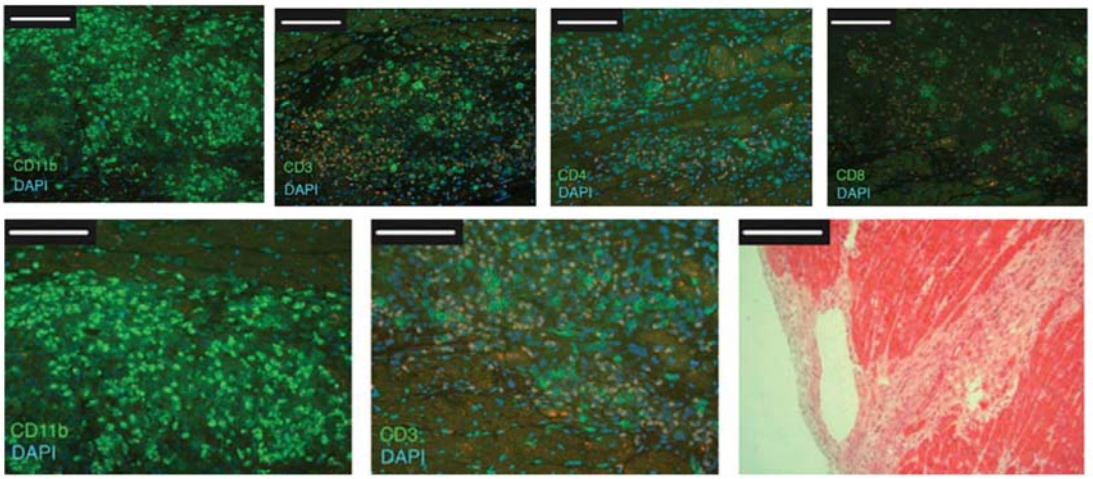


Figure 21. Immunological response to HESC transplanted into the myocardium of costimulation blockade treated C57BL/6 mice. Upper panel, this immune response was characterized by activated macrophages (CD11b+), T-cells (CD3+), where the T-cell response is characterized by both CD4+ cells and CD8+ cells after 1 month and in the lower panel, the same immune response was seen in one of the hearts (n= 1/6), 2 months after transplantation. In the majority of the hearts there was only a scar left in the myocardium two months after transplantation (n = 5/6). Bar represents: 100 µm in all panels apart from the lower right = 200µm.

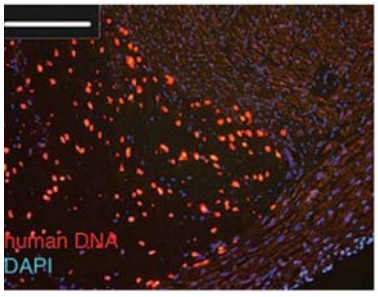


Figure 22. HESC 2 months after transplantation into the myocardium of C57BL/6 mice and received treatment with costimulation blockade directly after transplantation and then again 3 weeks later, which led to the acceptance of these cells in one recipient as demonstrated by the FISH positive red cells. Bar represents 200 µm.

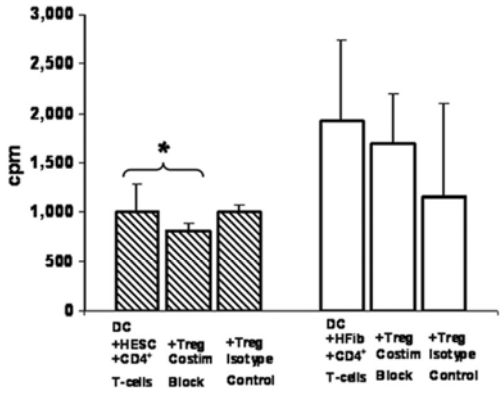


Figure 23. MLR showed that regulatory T-cells isolated from the costimulation blockade group down-modulated the immune response significantly, while regulatory T-cells isolated from the isotype control group did not.

8 GENERAL DISCUSSION

Cardiac regenerative cell-based therapy has emerged as a growing and promising future for therapeutic myocardial regeneration. Many challenges remain to be addressed before this approach can be applied in the clinic. One of the most important challenges is to elucidate which stem / progenitor cell is optimal for cardiac regeneration. By definition, the ideal cell population for cardiac cell-based therapy should be able to counteract the remodelling process that finally leads to heart failure.
At the time that we started our project, the concept of heart-derived cardiac progenitor cells had emerged as a new field that circumvents most of the problems related to allogeneic pluripotent stem cells (Barile et al., 2007). Instead of evaluating different differentiation protocols for generation of cardiomyocytes from pluripotent and multipotent stem cells, we focused on identifying the optimal cell type that could meet the above-mentioned definition and could function as a template for future generation of cardiac progenitors (CPCs) from pluripotent stem cells like HESCs.
Based on several previous reports that identified the LIM – homeodomain transcription factor Isl1 as a marker of multipotent undifferentiated cardiovascular progenitors residing in the heart (Cai et al., 2003; Sun et al., 2007), we have focused our research to characterize the Isl1 cardiac progenitors from the embryonic period to adulthood and how these cells respond to physiological and pathological stress. In this thesis a new concept of immunological tolerance to circumvent the immune rejection is presented. If clinical grade CPCs can be generated from HESCs appropriate cell quantities for cardiomyoplasty may be produced.

8.1 THE JOURNEY OF ISL1 CELLS ALONG THE LIFE SPAN

8.1.1 Spatial and time-dependent analysis of Isl1+ cells

When we started this research, most of our understanding about the molecular and cellular basis of developmental cardiogenesis was based on studies of rodent hearts, mainly mice. In these studies presented here, we have compared the embryogenesis in humans with rats. Taken in consideration, the program that governs the cardiac development in humans, however, differs significantly from the murine program. Murine cardiogenesis takes places in about 48 hours; meanwhile human cardiogenesis occurs over weeks, so the extrapolation of data should be taken with caution. Another issue is the heterogeneity, logistics and scarcity of human embryonic material, explaining why no time-adjusted systematic characterization could be performed on this material.

The role of the LIM homeodomain transcription factor Isl1 in the organogenesis of pancreas (Karlsson et al., 1990) and motor neurons of the ventral horn of the spinal cord has previously been well described (Tsuchida et al., 1994; Thor et al., 1999). Moreover previous studies have shown the importance of cardiac progenitors expressing Isl1 for the development of the embryonic heart (Cai et al., 2003; Yang et al., 2006; Sun et al., 2007). The genetic marking techniques used in these studies delineate specific cell types derived from Isl1+ cells, but they do not show the actual distribution of cells actively expressing the protein Isl1. In spite of that the

recombinase–based fate mapping technique is considered an important strategy for defining progenitor descendant relationships. However the interpretation of these data is a complex process which could explain the diverse results concerning the contribution of Isl1+ cells and their derivates to the first (FHF) and second heart fields (SHF) or both (Prall et al., 2007; Ma et al., 2008). To circumvent these problems in this thesis an Isl1+ cell is defined as a cell expressing the protein Isl1.

In *paper II*, using a longitudinal rat study, we determined the distribution and level of differentiation of cells expressing the protein Isl1, from the embryonic period to adulthood (Genead et al., 2010a). Analysis was initiated at GD 11 in rats, which corresponds to GD 9 in mice at which time point growth retardation has been observed in Isl1 mutant mice (Cai et al., 2003). Isl1 expressing cells were identified along the entire life span from the embryonic period (GD 11-15), during which the majority of the cardiac morphogenesis takes place, until adulthood. During the early embryonic time period the Isl1+ cells were present in abundance in the para-cardiac tissue, including regions like pharyngeal foregut endoderm and splanchnic mesoderm, and to a lesser extent around the ventral and lateral aspects of the evolving trachea and the anterior cardinal veins. These areas are suggested to be the SHF and these findings support the idea that Isl1+ cells constitute the majority of the cells of the SHF (Cai et al., 2003; Buckingham et al., 2005). At GD 13 the Isl1+ cells were mainly found in the developing heart and less in the remnants of the SHF. This is consistent with the findings in mice where most of the Isl1 progenitors had migrated into the heart by GD 9 (Sun et al., 2007).

In *paper II*, the Isl1 protein expression differed with the age of the embryonic heart (Genead et al., 2010a). At the earlier stages of development, there was a smaller amount of Isl1+ cells present within the heart. These cells were mainly localized in the myocardial layer of the bulbotruncal region and truncus arteriosus and few scattered Isl1+ cells were also present in the common atrial chamber. At the later stages of development, more Isl1+ cells were observed in the proximal and distal outflow tract, at the junction between the OFT and the ventricles and to a lesser extent in the inflow part of the right atrium. No Isl1+ cells could be identified in the left ventricle (LV) at any developmental stage. These data are consistent with the previous reports based on retrospective clonal analysis and genetic fate-mapping studies in mouse embryos (Buckingham et al., 2005; Cai et al., 2003; Sun et al., 2007), which indicated that Isl1 marks cells in the developing heart originating from the SHF.

Few Isl1+ cells were also present in the postnatal rat heart, mainly as clusters in the OFT, and at the junction to the ventricular tissue. These results are in keeping with two reports. The first report by Laugwitz and coworkers, that described the presence of few Isl1 progenitors in the neonatal heart, mainly in the atria and the outflow tract of mouse, rat and human specimens (Laugwitz et al., 2005). In a second report by Amir and coworkers, human myocardial specimens obtained from the right ventricular outflow tract (RVOT) of children with congenital heart disease were studied (Amir et al., 2008). In these neonatal heart biopsies from infants not older than one postnatal week, very few Isl1+ cells were found, often no more than 1 to 2 cells per specimen. However, the interpretation of data should be taken with caution, because the samples were collected from neonatal hearts with congenital anomalies. Probably these Isl1

cells are developmental remnants of the embryonic progenitors since their distribution coincides with the cardiac structures known to evolving from the SHF.

In continuum with the Isl1 cells journey along the entire life span, the presence of Isl1+ cells in the adult heart of pregnant rats was observed for the first time (Genead et al., 2010a). This is contradictory to previous reports where the Isl1 expression disappeared shortly after birth (Laugwitz et al., 2005). However, recently Khattar and his colleagues confirmed our findings, where they reported the presence of two subpopulations of Isl1 expressing cells in the young adult heart of different strains of mice (Khattar et al., 2011).
In our study the Isl1+ cells were very few and scattered within the OFT. To keep in mind, these cells were observed in the hearts of pregnant rats, which raises the role of stress as a stimulus for up-regulating dormant endogenous cardiac progenitors. Another explanation might be that the adult heart contains a pool of cardiomyocytes that can change into a fetal phenotype including expression of Isl1 or re-enter the cell cycle upon stimulation. A recent report support the hypothesis that silent cardiac progenitors could be activated upon injury, during which Isl1-expressing cardiomyocytes could be detected throughout the ventricle of the adult newt hearts (Witman et al., 2011).

The distribution of Isl1+ cells in aborted human embryonic hearts (gestational age 5-9 weeks) was also studied, again looking at the protein expression (Genead et al., 2010b). In the human embryonic material, for the first time the distribution of Isl1+ cells was identified during the early first trimester (Genead et al., 2010b). This is the period during which major morphogenesis of the human heart takes place. The distribution of Isl1+ cells was in a time-dependent manner dynamically changing from clusters of Isl1+ cells, mainly located in the proximal and distal OFT at 5 weeks of gestation, to a wider distribution at 9 weeks, where the Isl1+ cells were present not only in the OFT but also in the right atrium, right ventricle and the interstitial septum. As in the rat model (Genead et al., 2010a), no Isl1+ cells were detected in the LV at any developmental time point. Even if the Isl1 distribution in humans has not been widely studied, there are few reports consistent with our data, Bu and co-workers reported that Isl1 cells were present in the right atria, outflow tract and the left atrial appendage in the late first and early second trimester (Bu et al., 2009). On the other hand, Amir and co-workers aimed to study the distribution of putative resident cardiac stem cells in neonatal human hearts, and used human embryonic hearts as controls (Amir et al., 2008). In this study the Isl1 expressing cells in the embryonic hearts were found as clusters in the myocardial layer of the right atrial wall but nothing in the OFT or the ventricular tissues (Amir et al., 2008). In another study by Alfakir and coworkers looking at Isl1 mRNA expression, demonstrated Isl1 in RV, LV, RA, LA and OFT six weeks post conception (pc) meanwhile at 11 weeks pc the Isl1 mRNA was mainly expressed in the OFT and LA (Alfakir et al., 2010). Even if these reports describe the discrepancies in the distribution of Isl1+ cells in the human embryonic hearts reported in the different studies, Isl1+ cells are present and contribute to the development of the human embryonic heart in a similar manner as previously described for rodents (Genead et al., 2010a; Cai et al., 2003). Next it is important to characterize the differentiation and proliferative capacity of the *in vivo* expressing Isl1+ cells.

8.1.2 The differentiating and proliferative capacity of Isl1+ cells

In *paper I* and *II*, the *in vivo* differentiation and proliferation profile expression of the Isl1+ cells were identified within the human and rat embryonic hearts. Only a few of the Isl1 expressing cells were in an undifferentiated stage, while the majority were in a differentiated stage, expressing smooth muscle and cardiomyocyte markers (Genead et al., 2010a; Genead et al., 2010b). Several reports in mouse and human support these findings, describing a down-regulation of the Isl1 expression after migration into the heart when the Isl1+ cells start to differentiate and contribute to the constituents of the heart i.e. cardiac myocytes, smooth muscle and endothelial cells during heart development (Cai et al., 2003; Sun et al., 2007; Bu et al., 2009). Furthermore, in studies by Kruithof and coworkers, they revealed that the OFT myocardium consists of relatively undifferentiated CPCs (Kruithof et al., 2003). This balance between the fully differentiated and undifferentiated cell population may be crucial during heart development, leaving an endogenous reservoir of cells with high plasticity.

Unexpectedly both in rat and human embryonic hearts only few of the Isl1+ were showing signs of cell cycle activity (Genead et al., 2010a; Genead et al., 2010b). Instead, in the embryonic heart, the cells that demonstrated the highest proliferative capacity were the cardiomyocytes (Genead et al., 2010a). According to the results by Cai et al, in mice the Isl1 expression was down-regulated as soon as the Isl1+ cells started to differentiate into cardiomyocytes (Cai et al., 2003). In contrast, in our embryonic rat study, Isl1 protein was expressed together with cardiomyocyte and smooth muscle markers (Genead et al., 2010a). In the postnatal period, there were still undifferentiated Isl1+ cells in the OFT, some of them were proliferating and others also expressing the mature cardiomyocyte (TnT) marker. Even in the adult heart we could identify a few Isl1+ cells, all of which had also expressed cardiomyocyte marker TnT. No undifferentiated Isl1+ cells were present in the adult heart.

To discuss further the issue of *in vivo* dynamics of proliferation in both the embryonic and the postnatal heart, several aspects should be highlighted.
First as regards the pattern of cell proliferation in the embryonic period, there is an initial period of rapid cell proliferation at about E8 in mouse (Pasumarthi and Field, 2002; Alsan and Schultheiss, 2002) then a transiently slower phase of proliferation, and finally a second peak of proliferation at E11 (Pasumarthi and Field, 2002). However, the molecular mechanisms regulating these waves of proliferation within the cardiac mesoderm are poorly understood, concerning which cells (the progenitor pool or the embryonic cardiomyocytes) are responsible for proliferation.
In *paper I*, the majority of the ventricular cardiomyocytes expressed the cell-cycle associated nuclear protein, Ki67, while unexpectedly only a few Isl1+ cells co-expressed the same marker in their areas of preferred distribution, namely the outflows tract (Genead et al., 2010a). This is consistent with findings observed during the embryonic heart development, where different cardiac subdomains demonstrated different proliferation patterns. Increased levels of cell proliferation were observed in the myocardium of the atria and ventricles, in comparison to the OFT (Christoffels et al., 2000).

A recent report by Walsh and co-workers, combining FACS sorting and immunostaining for BrdU uptake or Ki-67 indicated that a range of 12–23% (depending on different methods) of embryonic cardiomyocytes were positive for cell-cycle markers. This activity declined rapidly in the postnatal period (Walsh et al., 2010). In *paper II*, two markers instead of one were used to evaluate the proliferative capacity of the studied cardiac cells. A rather wide range of difference in the percentage of the positive cells expressing the two markers (Ki67 or phosphorylated H3) were observed, consistent with data obtained from Walsh and co-workers.
Several reports support the possibility that fully differentiated cardiomyocytes within the developing myocardium continue to proliferate, co-expressing phospho-histone H3, indicating that even fully differentiated cells have a dividing capacity as they undergo full differentiation (Goetz et al., 2006; Pasumarthi and Field, 2002). The same applies for many other tissues such as the skeletal muscle and the nervous system, in which cell cycle withdrawal is tightly associated with the onset of terminal differentiation (Alexiades and Cepko, 1996; Walsh and Perlman, 1997).

In our model we reported only few Isl1+ progenitor cells within the embryonic heart co-expressing markers of cell proliferation. Consistent with these observations, a report by Amir and colleagues demonstrated several populations of CPCs in the human fetal and early infant myocardium and evaluated their rate of proliferation (Amir et al., 2008). They showed that the majority of differentiated cardiomyocyte co-expressed Ki67 during the fetal period (40-70%), declining rapidly in the postnatal period. In contrast, only few c-Kit progenitors and early cardiomyocyte progenitors expressing NKx2.5 expressed the Ki67 in the fetal heart, which further decreased after birth (Amir et al., 2008). Taken altogether, the unexpected findings of few Isl1+ cells expressing Ki67 were consistent with reported findings that only few c-Kit+ cells expressed Ki67 in the fetal and early postnatal period (Amir et al., 2008).

Another methodological aspect should be considered regarding the use of Ki67, which is considered as a marker of cell-cycle activity, indicating that the cells are not in the G0 phase of cell cycle. However this does not mean that they undergo active cellular division (cytokinesis). Several reports based on Ki67 positive cardiomyocytes might reflect a nuclear division (karyokinesis) without concurrent cytoplasmic division (Beltrami et al., 2001; Meckert et al., 2005).
In our model we analysed cellular proliferation using Ki67 on *in situ* sections at a certain time point, which is not the optimal. This means that it was difficult to trace the proliferative pattern of our cells of interest i.e. the Isl1 in a real-life imaging. However, the *in vitro* model from the embryonic heart material revealed a rather modest degree of proliferation, which was similar to our *in vivo* results (data not shown).
To label the isolated Isl1 cells *in-vivo* and trying to trace them under different conditions would be of great importance to study their dynamics. Another aspect would be to isolate the Isl1 cells from the tissue and study them *in vitro*. This is under investigation in our laboratory.

From the above discussions concerning the Isl1 journey, it is hypothesized that during cardiogenesis, the pool of Isl1+ cells, while migrating into the developing heart from their extra-cardiac regions, undergoes an active conversion from a multipotent stage to a more lineage restricted stage. At the same time, the Isl1 expression is down-

regulated due to differentiation into cardiomyocytes, and their proliferative capacity is turned down (Figure 24).

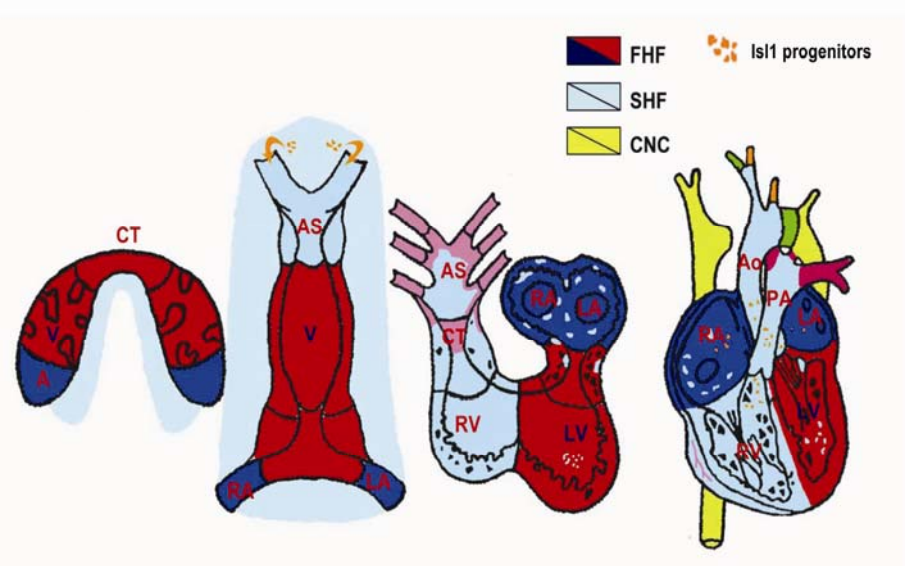


Figure 24. Schematic illustration showing the mammalian embryonic heart development and the proposed role of the Isl1 progenitors**.**
FHF: first heart field; SHF: second heart field; CNC: cardiac neural crest; CT: conotruncal; AS: aortic sac; RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium; Ao: Aorta; PA: pulmonary artery; V: Common ventricle; A: common atria.

In order to study the *in vitro* proliferating and differentiating capacity of embryonic cardiomyocytes cultures from human embryonic hearts were generated (Genead et al., 2010b). In culture the embryonic cardiomyocytes developed into spontaneously beating cardiospheres (CSs). The CSs contained a heterogeneous population of cardiomyocytes at different level of differentiation. The majority of the cells expressed cardiomyocyte markers like TnT, representing more mature cells. The CSs also contained a mixture of early cardiomyocyte progenitors expressing Nkx2.5 but there were only a few Isl1+ cells present. In electron microscopy the cells contained contractile elements, mitochondria and formed gap junctions with the surrounding cardiomyocytes. The CSs also responded to external stimuli like electrical stimulation and β-adrenergic stimulation by reducing the field potential duration and decreasing the inter-beat interval, thus beating with a higher frequency. Consistent with these data, other studies have reported CSs that were obtained from rodents, pigs and human biopsy of adult hearts. These CSs demonstrated a similar heterogeneity of cells (Messina et al., 2004; Miyamoto et al., 2010; Smith et al., 2007).
The generation of 3D CSs for implantation purposes represents a potential promising strategy for cardiomyoplasty. The 3D structure closely resembles the "niche" in terms of interaction among different cells and their supporting matrix, which should improve survival of the cells after implantation. Recent studies in mouse and pigs, revealed greater benefits of 3D cardiospheres in comparison to the regular 2D structures when transplanted into an ischemic model (Lee et al., 2011; Li et al., 2010). Therefore it was important to show that the generated human CSs responded to external stimuli, otherwise there should be a risk of arrhythmia after implantation.

Moreover our results can provide the basis for development of an *in vitro* system with beating cardiomyocytes for evaluating the cardiotoxicity of different drugs, thus preventing the development congenital malformations (Danielsson, 2010).
The CSs used in our study were derived directly from the culturing of early embryonic cardiomyocytes and cardiac progenitors (Genead et al., 2010b). The optimal situation would be if it was possible to isolate the Isl1+ progenitors, expand them *in vitro* and then differentiate them into spontaneously beating cardiospheres. A recent study that supports this idea has employed fluorescence-marked transgenic embryo and embryonic stem cells as well as combinations of positive and negative fluorescence activated cell sorting to document the presence of FHF and SHF progenitors with distinctive transcriptional profiles (Domian et al., 2009). They showed that the transcriptional Isl1 / Nkx2.5 signature gave rise to a spontaneously beating three dimensional muscular film.
An option would be to determine a specific profile of surface markers expressions of Isl1+ cells of the human embryonic heart. Then a similar strategy might be used for generating beating cardiomyocytes for cardiomyoplasty. Another strategy would be to generate Isl1+ cells from HESCs and implant them using tolerance induction to prevent immune rejection. Since Isl1+ cells seem to be present even in the adult heart, it might even be possible to stimulate the endogenous regenerative capacity and thereby inhibit the remodelling process that follows heart failure development.

8.2 CPCs TURNOVER IN RESPONSE TO STRESS

Paper II is the first report on the detection of scattered Isl1+ cells within the outflow tract of adult pregnant rat heart (Genead et al., 2010a). The hypothesis was that pregnancy, as a kind of physiological stress, plays a role in altering the dynamics and turnover of cells within the adult heart. In accordance with this hypothesis, an experimental design was set up to explore how cardiac regeneration and cell turnover adapted to various stress modalities like pregnancy, myocardial infarction and ischemia-reperfusion with or without the growth factors IGF-1 and HGF (Genead et al., 2011).

Previous reports have revealed that the adult heart is a highly dynamic organ in which cardiac homeostasis can be modulated under various physiological or pathological states (Kajstura et al., 2010; Nadal-Ginard et al., 2003). Our study systematically examined for the first time the time-dependent and spatial up-regulation of the best characterized cardiomyocyte progenitor markers c-kit and Isl1, as well as the early cardiomyocyte marker Nkx2.5, during experimental pathological and physiological stress stimuli in adult rat heart (Genead et al., 2011). Observations were based on mRNA and protein analysis of tissue specimens from the different cardiac sub-domains. In this way, the expressions of Isl1, c-Kit and Nkx2.5 were verified by two independent methods.
In order to compare the up-regulation of our target marker Isl1, we chose another well studied progenitor marker namely c-Kit. This is the tyrosine kinase receptor for the stem cell factor (SCF) and was initially reported as a hematopoietic stem cell marker, with controversial data regarding their cardiomyogenic *in vivo* potential (Lyman and Jacobsen, 1998). c-Kit+ cells as resident progenitors in the heart were first reported in

2003 (Beltrami et al., 2003). Beltrami and co-workers succeeded to isolate c-Kit+ /Lin-/CD45- from rats and demonstrated their potential for myocardial regeneration by differentiating them into all the cardiac lineages and regenerating infarcted myocardium. Furthermore, c-Kit+ cells have also been isolated from human hearts, and upon transplantation into infarcted myocardium of immunodeficient rats, they generated a chimeric heart, with new population of cardiomyocytes and vasculature (Bearzi et al., 2007; Beltrami et al., 2003). Another advantage of using these cells for cardiomyoplasty is that in culture c-Kit+ cells exhibit long term stability, self renewal and clonogenic properties (Miyamoto et al., 2010). However, the origin and phenotype of the c-kit+ cells in the adult heart is still under debate. All c-Kit+ cells are not stem cells since c-Kit can also be expressed on mast cells or other hematopoietic cells (Baghestanian et al., 1997; Pouly et al., 2008; Sandstedt et al., 2010; Zhou et al., 2010).

Among the different stimuli, ischemia-reperfusion injury induced the strongest up-regulation of both c-Kit and Nkx2.5. This up-regulation was time-dependent and was detected throughout the entire heart. The up-regulation of c-Kit could not solely be explained by activation of mast cells in the heart, since the c-Kit expression did not correlate to the changes of mast cells expression.
Whether the stem / progenitor cells in general, represent different subpopulations or whether they derive from a common precursor have not been resolved. In *paper III*, there was a spatial mismatch on one hand of c-Kit and Nkx2.5 and on the other Isl1 expression, indicating their different dynamics and origins. Both c-Kit and Nkx2.5 were globally up-regulated, while Isl1 up-regulation was localized to the outflow tract, where these cells previously have been demonstrated to reside in the rat and human embryonic hearts (Genead et al., 2010a; Genead et al., 2010b).
Consistent with these findings, several reports have revealed the existence of two different subpopulations of cells expressing c-kit and Isl1 (Pouly et al., 2008; Laugwitz et al., 2005). Moreover, Itzhaki-Alfia and co-workers isolated viable heart cells, from patients during heart surgery or as endomyocardial biopsies. These cells expressed both c-Kit and Isl1 and the best source was from the right atrium of females. *In vitro*, no overlap was observed between these two markers, indicating the existence of two subpopulations and the possible role of stress in altering the dynamic expression of markers (Itzhaki-Alfia et al., 2009).

In *paper III*, interestingly, ischemia-reperfusion (IR) injury was the strongest stimulus for the up-regulation of both c-Kit and Isl1. In contrast to c-Kit, Isl1 was not up-regulated by pregnancy or myocardial infarction. Consistent with these data Hsieh and co-workers, based on a genetic fate-mapping study, revealed that there was an increased turn-over of cardiomyocytes in adult mice with ischemia and pressure overload (Hsieh et al., 2007). Which progenitor cell markers were associated to this endogenous turn-over of cardiomyocytes were not studied. Kubo and co-workers reported an increasing abundance of c-Kit cardiac progenitors in patients with advanced heart failure regardless of the underlying etiology (Kubo et al., 2008). These observations may suggest that some factors are released during these pathological states that induce the up-regulation of cardiac progenitors stimulating the regeneration of new cardiomyocytes. Alternatively, the stress on the heart may induce a fetal cardiomyocyte phenotype with up-regulation of markers such as Nkx2.5, c-Kit and Isl1.

It was interesting to study if the endogenous IGF-1 and HGF are involved in the up-regulation of cardiac progenitor markers in the IR model and if exogenous local administration of these growth factors could further potentiate this effect. Based on previous studies, the role of these growth factors as cytoprotective, angiogenic and anti-apoptotic effects have been established (Torella et al., 2004; Wang et al., 2004). We concluded that the endogenous up-regulation of IGF-1 and HGF followed the same pattern as for the studied cardiac progenitor markers. The addition of IGF-1 and HGF could further boost the endogenous expression of these growth factors which subsequently further stimulated the expression of Isl1, especially in the outflow tract, but did not further affect the expression of c-Kit. A recent study by Ellison and co-workers revealed that the intracoronary injection of IGF-1/HGF activated the endogenous cardiac stem cell pool and improved the cardiomyocyte survival in a pig model (Ellison et al., 2011). Similar effect applies when CPCs were injected together with IGF-1 bound to self-assembling peptide nanofibers in the infarction border zone, which stimulated cardiomyogenesis (Padin-Iruegas et al., 2009).

This concept actually opens up a new field. It might be possible to stimulate and thereby augment the endogenous regenerative capacity of the heart. This offers distinct advantages to stem cell implantation since the problems with engraftment and immune rejection are avoided.

8.3 IS IT POSSIBLE TO INDUCE IMMUNE TOLERANCE TOWARDS HESCs?

Cardiac regenerative medicine is a potential alternative to conventional therapies. However several challenges need to be overcome, one being the prevention of immunological rejection. In this thesis, focus has been on cardiac progenitors as potential cell source for myocardial regeneration. A potential source for large amounts of progenitor cells is embryonic stem cells. In order to test the immunological aspect of cell transplantation of such cells and due to a lack of a sufficiently pure population of Isl1 progenitors, undifferentiated HESCs were chosen for the study of immune tolerance.

In the clinical setting, despite the great potential of HESCs, several challenges concerning their immunogenicity have not been fully addressed and strategies to avoid rejection remain largely unsolved. Conflicting data exist concerning whether the HESCs are immune privileged or not. Early reports indicated that HESCs could be immune privileged (Drukker et al., 2002; Li et al., 2004), because they do not express HLA class II molecules and show limited expression of HLA class I molecules. However, recent studies showed that HESCs can induce immune rejection in xenogeneic transplantation (Grinnemo et al., 2006; Swijnenburg et al., 2008). Thus to overcome the immunogenicity of transplanted HESCs, an immunomodulatory regimen is necessary. Preferably the ideal immunosuppressive therapy should involve only a brief period of immunosuppression but be able to induce specific and long-term tolerance to the donor cells with limited side-effects (Chidgey et al., 2008).
The strategy of blocking costimulatory molecules at the time of transplantation has emerged as a promising strategy. This can be achieved by treating the recipient with

monoclonal antibodies and receptor specific fusion proteins the first week after transplantation. The most well characterized are the B7-CD28 family (Greenwald et al., 2005; Sharpe and Freeman, 2002), CD40-CD40L (TNF receptor family) (Croft, 2003) and LFA-1 (Van Seventer et al., 1990; Zuckerman et al., 1998). The combined use of anti-CD40ligand (CD40L) and cytotoxic-T-lymphocyte-associated antigen 4 (CTLA4Ig), which blocks B7 signaling, showed synergistic effects on the induction of transplantation tolerance in rodents and nonhuman primates (Larsen et al., 1996; Kirk et al., 1997). LFA-1 is essential to the formation of the immunological synapse as well as the trafficking and costimulation of T-cells. By adding anti-LFA-1 to anti-CD40L and CTLA4Ig synergistic effects are achieved (Larsson et al., 2003).

In *paper IV*, this triple costimulation blockade regimen (CTLA4Ig/ anti-CD40L/ anti-LFA-1) was tested in a mouse model, to evaluate the potentiality to induce immune tolerance to transplanted HESCs (Grinnemo et al., 2008). The costimulation blockade was sufficiently robust to induce tolerance to undifferentiated HESCs in the immune-privileged environment of the testis (Nasr et al., 2005) and to induce regulatory T-cells to HESCs when transplanted into the myocardium of immunocomptent mice. These results are in keeping with the ability of the costimulation blockade to induce tolerance to cardiac allografts (Larsen et al., 1996), pancreatic islets (Lenschow et al., 1992; Kumagai-Braesch et al., 2007), and bone marrow transplants (Kurtz et al., 2009).

In *paper IV*, in contrast to HESCs transplanted to the testis, no surviving cells could be seen when transplanted into the myocardium of costimulation blockade treated mice. When treated with a booster dose of the costimulation blockade, HESC-derived cells with a teratoma-like structure were found in only one out of five mice. Transplanted HESCs induce an acute CD4+ T-cell mediated rejection which is inhibited by the blockade of costimulatory molecules. This leads to a state of non-reactivity in the helper T-cell population which leads to the generation of immunological memory. This memory is maintained by regulatory T-cells (CD4+/CD25+/Foxp3+) specific to the undifferentiated HESCs. These regulatory T-cells then inhibit new naive T-cells which are potentially reactive towards HESC and thereby prevent rejection in the future.

Immunological self-tolerance can be either central (thymic) tolerance or peripheral (non-thymic) tolerance. Peripheral tolerance is a consequence of inappropriate costimulation of T-cells after having exited the thymus resulting in T-cell anergy or non-reactivity. Some of these anergic T-cells differentiate into regulatory T-cells (Miyara and Sakaguchi, 2007). Regulatory T-cells have been linked to the induction of transplantation tolerance in preclinical studies (Joffre et al., 2008) and to stable renal-allograft function (Kawai et al., 2008).
In *paper IV*, the existence of the regulatory T-cells in the testis support the hypothesis that costimulation blockade induced peripheral tolerance similar to other studies using the same protocol (Kumagai-Braesch et al., 2007). The isolated regulatory T-cells from the costimulation blockade group down-regulated the immune response induced by HESCs *in vitro*. This effect was specific to HESCs and not human fibroblasts, indicating that the recipient became immunologically tolerated towards the undifferentiated HESCs.

A recent study by Pearl and co-workers showed that blocking leukocyte costimulatory molecules permit ESC engraftment. They have demonstrated the success of this immunosuppressive therapy for undifferentiated mouse and human ESCs, iPS as well as more differentiated ESCs derivatives. In addition, they showed that the tolerance induced by the costimulation blockade is antigen-specific consistent with our findings described above (Pearl et al., 2011).

These findings are promising but still many challenges and questions need to be clarified before proceeding to the clinic. Attempts to use anti-CD40L in a clinical setting were unsuccessful because of thromboembolic events complicating their administration (Sidiropoulos and Boumpas, 2004). As an alternative to overcome this problem, humanized mouse antihuman CD40Ab has been developed and tested in nonhuman primates and demonstrated allograft survival (Haanstra et al., 2003).

9 CONCLUSIONS

- The human embryonic heart can be a potential source of cardiac progenitor cells.

- Clusters of Isl1+ cells are present in the early first trimester human embryonic heart. Some Isl1+ clusters are undifferentiated while others express cardiomyocyte and smooth muscle lineage markers. Spontaneously beating cardiospheres from the human embryonic heart responded to electrical and pharmacological β-adrenergic stimulation. This is an important characteristic if these cells are to be used clinically.

- During embryogenesis in a longitudinal rat model, Isl1 cells seem to migrate from extra-cardiac regions into the proximal part of the heart, proliferating and giving rise to cardioblasts. Part of the Isl1+ cell pool persists into adulthood.

- Ischemia-reperfusion injury among other stress modalities was the strongest stimulus for up-regulation of cardiac progenitor cell markers suggesting activation of an endogenous cardiomyocyte regenerative reserve, correlating to up-regulation of endogenous IGF-1 and HGF. There was correlation between the up-regulation of the both progenitor cell markers Isl1 and c-Kit and the up-regulation of the early cardiomyocyte marker Nkx2.5.

- A short course of costimulation blockade induced long-term tolerance to undifferentiated HESCs in the immune-privileged environment of the testis and induced regulatory T-cells specific to the undifferentiated HESCs when transplanted into the myocardium of immunocompetent mice. A booster dose of costimulation blockade enhanced xenograft graft survival in the myocardium.

10 FUTURE PERSPECTIVES

- The existence of the Isl1+ cells was demonstrated in the human embryonic heart. So it is worthy to consider the human embryonic material as a potential source for cardiac progenitors from which a relatively homogenous population of the Isl1 CPCs could be expanded *in vitro* for future use in cardiomyoplasty.

- The ability to develop the cardiospheres from human embryonic hearts could open new windows for using these promising 3D structures in transplantation studies and evaluate their role in improving cardiac functions.

- Isl1 cardiac progenitors are considered as promising endogenous cardiac progenitors. In order to get a pure population of thee Isl1+ cells isolation is mandatory. However, this is not applicable at this moment because Isl1 is a nuclear transcription factor. Isolation is usually based on the expression of surface markers so one possibility would be to identify a cell surface marker profile that specifically identifies Isl1 cells. As part of such a strategy, these cells need to be further characterized both at the transcriptional and protein levels. In the next step it might be possible to expand and subsequently differentiate the Isl1 population *ex vivo* for future use in clinical trials.

- The concept of myocardial regeneration by means of stimulating or augmenting the endogenous regenerative potential is an attractive approach and more easily applicable in the clinic than the administration of stem cells. In *paper III*, our preliminary results could demonstrate the up-regulation of the studied cardiac progenitor markers as a response to different kinds of stress. Exogenous administration of growth factors potentiated these up-regulations. Several options are warranted to be tested in order to elucidate the mechanistic background and underlying mediators of such a regenerative response. In the next step these growth factors could be utilized to boost the endogenous regenerative capacity seen in patients with ischemic heart failure and thereby circumvent the need for stem cell transplantation. Another aim is to evaluate the effectiveness of the paracrine response induced by different stem cells, like mesenchymal stem cells.

- The promising results concerning the immune tolerance induced by short term use of costimulation blockade could open a new window to test this regime in transplantation studies on other cell types like Isl1+ cells.

11 ACKNOWLEDGMENTS

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